

Graduate School for Cellular and Biomedical Sciences

University of Bern

**Development of a novel marker vaccine
platform for protection against Bluetongue
Virus (BTV)**

PhD Thesis submitted by

Stefanie Kochinger

from **Austria**

for the degree of

Doctor of Veterinary Medicine and Philosophy (DVM, PhD)

Supervisor

PD. Dr. Gert Zimmer

Institute of Virology and Immunology

Vetsuisse Faculty of the University of Bern

Co-advisor

Prof. Dr. Eliane Marti

Division of Experimental Clinical Research

Vetsuisse Faculty of the University of Bern

Original document saved on the web server of the University Library of Bern



This work is licensed under a
Creative Commons Attribution-NonCommercial-No derivative works 2.5 Switzerland
licence. To see the licence go to <http://creativecommons.org/licenses/by-nc-nd/2.5/ch/> or
write to Creative Commons, 171 Second Street, Suite 300, San Francisco, California 94105,
USA.

Copyright Notice

This document is licensed under the Creative Commons Attribution-Non-Commercial-No derivative works 2.5 Switzerland. <http://creativecommons.org/licenses/by-nc-nd/2.5/ch/>

You are free:



to copy, distribute, display, and perform the work

Under the following conditions:



Attribution. You must give the original author credit.



Non-Commercial. You may not use this work for commercial purposes.



No derivative works. You may not alter, transform, or build upon this work..

For any reuse or distribution, you must take clear to others the license terms of this work.

Any of these conditions can be waived if you get permission from the copyright holder.

Nothing in this license impairs or restricts the author's moral rights according to Swiss law.

The detailed license agreement can be found at:

<http://creativecommons.org/licenses/by-nc-nd/2.5/ch/legalcode.de>

Accepted by the Faculty of Medicine, the Faculty of Science and the
Vetsuisse Faculty of the University of Bern at the request of the
Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

TABLE OF CONTENT

TABLE OF CONTENT	4
ABSTRACT	5
ABBREVIATIONS	7
INTRODUCTION BLUETONGUE	8
History and Epidemiology	8
Bluetongue disease	10
Structure and replication cycle	12
Vaccine strategies	14
INTRODUCTION VSV REPLICONS	16
Vesicular stomatitis virus structure and replication	16
Vesicular stomatitis virus replicon in experimental vaccines	17
Generation of VSV replicon particles expressing foreign antigens	18
AIM OF THE PROJECT	19
RESULTS	20
Unpublished results	20
Published results	29
CONCLUSION AND OUTLOOK	38
ACKNOWLEDGEMENT	42
<i>CURRICULUM VITAE</i>	43
LIST OF PUBLICATIONS	44
DECLARATION OF ORIGINALITY	45
REFERENCES	46

ABSTRACT

Bluetongue virus (BTV) is an economically important member of the genus Orbivirus and closely related to African horse sickness virus (AHSV) and Epizootic hemorrhagic disease virus (EHDV). Currently, 26 different serotypes of BTV are known. The virus is transmitted by blood-feeding *Culicoides* midges and causes disease (bluetongue [BT]) in ruminants. In 2006/2007, BTV serotype 8 (BTV-8) caused widespread outbreaks of BT amongst livestock in Europe, which were eventually controlled employing a conventionally inactivated BTV vaccine. However, this vaccine did not allow the discrimination of infected from vaccinated animals (DIVA) by the commonly used VP7 cELISA.

RNA replicon vectors based on propagation-incompetent recombinant vesicular stomatitis virus (VSV) represent a novel vaccine platform that combines the efficacy of live attenuated vaccines with the safety of inactivated vaccines. Our goal was to generate an RNA replicon vaccine for BTV-8, which is safe, efficacious, adaptable to emerging orbivirus infections, and compliant with the DIVA principle.

The VP2, VP5, VP3 and VP7 genes encoding the BTV-8 capsid proteins, as well as the non-structural proteins NS1 and NS3 were inserted into a VSV vector genome lacking the essential VSV glycoprotein (G) gene. Infectious virus replicon particles (VRP) were produced on a transgenic helper cell line providing the VSV G protein *in trans*. Expression of antigens *in vitro* was analysed by immunofluorescence using monoclonal and polyclonal antibodies.

In a pilot study, sheep were immunized with two different VRP-based vaccine candidates, one comprising the BTV-8 antigens VP2, VP5, VP3, VP7, NS1, and NS3, the other one containing antigens VP3, VP7, NS1, and NS3. Control animals

received VRPs containing an irrelevant antigen. Virus neutralizing antibodies and protection after BTV-8 challenge were evaluated and compared to animals immunized with the conventionally inactivated vaccine. Full protection was induced only when the two antigens VP2 and VP5 were included in the vaccine.

To further evaluate if VP2 alone, a combination of VP2 and VP5 or VP5 alone were necessary for complete protection, we performed a second animal trial. Interestingly, VP2 as well as the combination of VP2 and VP5 but not VP5 alone conferred full protection in terms of neutralizing antibodies, and protection from clinical signs and viremia after BTV-8 challenge.

These results show that the VSV replicon system represents a safe, efficacious and DIVA-compliant vaccine against BTV as well as a possible platform for protection against other Orbiviruses, such as AHSV and EHDV.

ABBREVIATIONS

BTV	Bluetongue virus
VSV	Vesicular stomatitis virus
VRP	Virus replicon particles
GFP or *	Green fluorescent protein
IFT	Immunofluorescent test/assay
MOI	Multiplicity of infection
NS	Non-structural protein
VP	Viral protein
G	Glycoprotein
Δ G	lacking Glycoprotein
AHSV	African horse sickness virus
EHDV	Epizootic hemorrhagic disease virus
RNA	Ribonucleic acid
kDA	kiloDalton
ELISA	Enzyme linked immunosorbent assay
dsRNA	double-stranded ribonucleic acid
kb	kilobases

INTRODUCTION BLUETONGUE

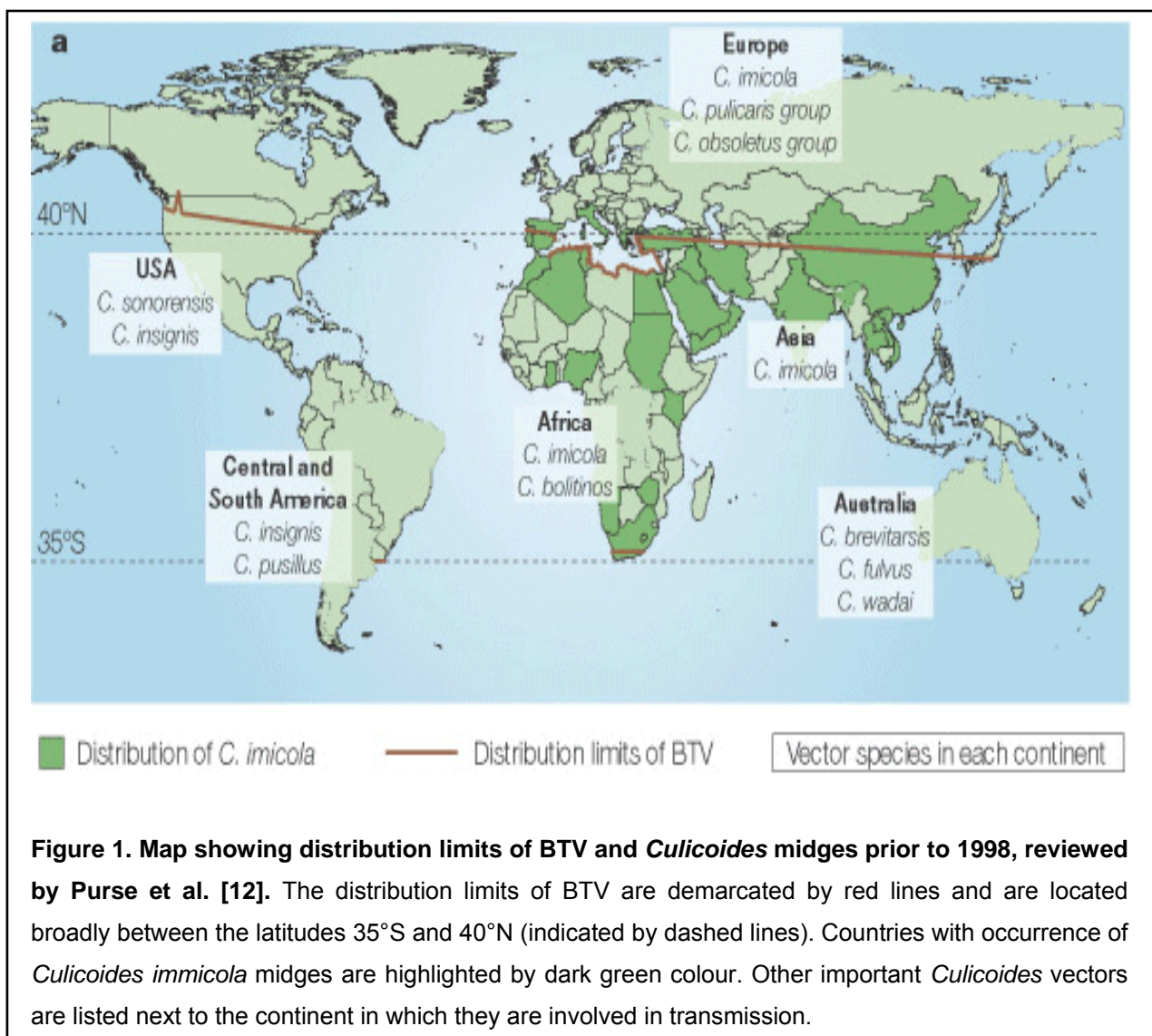
Bluetongue virus (BTV) is an *Orbivirus* of the *Reoviridae* family, and the causative agent of a haemorrhagic disease (bluetongue [BT]) in ruminants, mainly sheep [1]. There are currently 26 recognised serotypes of BTV, which are transmitted by blood-feeding *Culicoides* midges and sometimes vertically in animals [2, 3]. BTV is closely related to African horse sickness virus (AHSV).

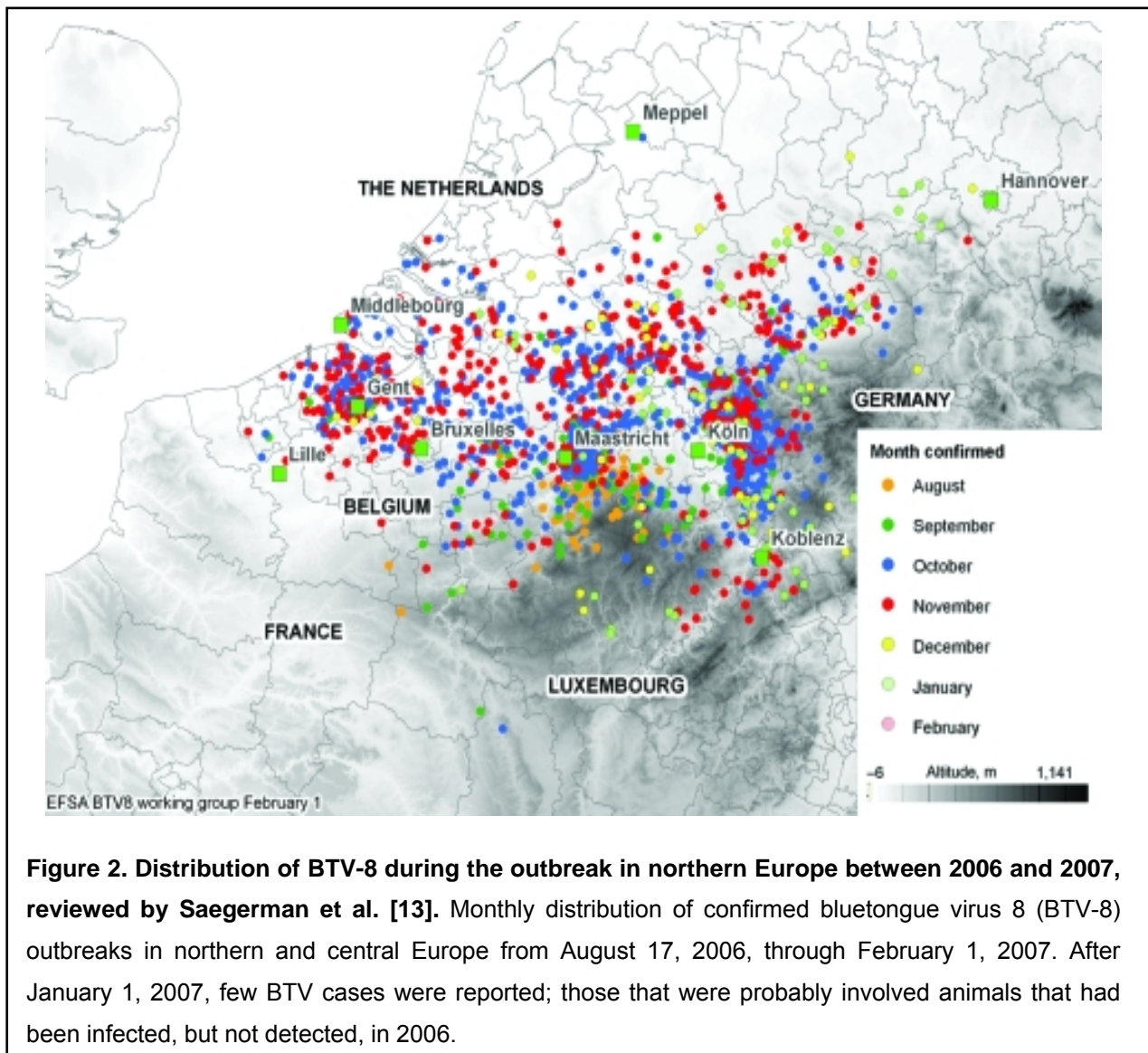
History and Epidemiology

Bluetongue was first described in Merino sheep in the late 19th century in South Africa and got its name due to severe cyanosis of the tongue of some severely affected sheep [4]. Later in the 1990s, it was determined that BT was a disease of ruminants occurring worldwide from latitudes of approximately 40 degrees North to 35 degrees South [5]. Furthermore, the disease was responsible for considerable economic losses especially at the incursional margins of the global range of BTV infection [6] (**Figure 1**). Although BTV had transiently made brief incursions into Southern Europe, these did not persist in the continent [5]. However, between 1998 and 2001, eight BTV strains of five different serotypes (BTV-1, 2, 4, 8, 9 and 16) emerged in several countries around the northern rim of the Mediterranean Basin where they had previously not been recorded, likely because of climatic changes which allowed overwintering of the virus in *Culicoides* vector midges [7].

A novel strain of BTV serotype 8 (BTV-8), which had never previously been detected in Europe, emerged in 2006 as an epidemic wave in Western and Northern Europe [4, 8, 9]. This outbreak had a significant economic impact, not only because of morbidity and mortality caused by the disease in sheep and cattle, but also because

of restrictions imposed on livestock movement and trade [10]. In August 2006, BTV-8 was detected in sheep in the Netherlands, followed by Germany, Belgium and France. In 2007, the outbreak was confirmed in Switzerland, the United Kingdom, Luxembourg, Denmark and the Czech Republic. In 2008, Spain, Italy, Greece and Austria also detected BTV-8 infection in cattle and sheep. It remains uncertain precisely how BTV-8 arrived in Northern Europe, but the absence of outbreaks in southern parts of the continent points towards a direct entry mechanism rather than a linear extension from its ancestor, potentially a Western lineage virus from sub-Saharan Africa [11].





Bluetongue disease

BTV is transmitted to livestock by blood-feeding *Culicoides* midges, in Europe mainly by *C. imicola*, *C. pulicaris* and *C. obsoletus*. In cattle, goats, and wild ruminants, BTV infection is typically asymptomatic despite prolonged viremia. These host species represent a potential reservoir for unnoticed dissemination of BTV in ruminant populations. In sheep, however, BTV infection often results in an acute disease with associated high morbidity and mortality, depending on the virulence of the virus and

the sheep breed affected [14]. Following an initial replication in the lymph nodes, BTV targets endothelium and mononuclear phagocytes [15]. After an incubation period of 2 to 15 days, clinical signs in the acute phase of disease comprise high fever, salivation, depression, nasal discharge, hyperaemia and congestion of the muzzle, lips, face, eyelids and ears leading to tissue oedema (**Figure 3 A**). Ulcerations and necrosis of the mucosae of the mouth are often observed, the tongue may become hyperaemic and oedematous. Lameness due to coronitis, pneumonia or lung oedema can be observed in severe cases. In cases with fatal outcome, sheep usually die after 8 to 10 days.

Certain strains of BTV, notably the northern European strain of BTV-8, can cross the placental barrier, leading to infection of the developing fetus [3]. Hence, abortions and malformations of offspring are frequently associated with infection of pregnant animals with certain strains of the virus [16-18].

Frequently observed pathological findings comprise hemorrhages at the base of the pulmonary artery (A. pulmonalis), the papillary muscles of the left ventricle of the heart, enlarged haemorrhagic lymph nodes, erosions on ruminal pillars, and ulcers of the mucosal lining throughout the upper gastrointestinal tract (**Figure 3 B-F**).

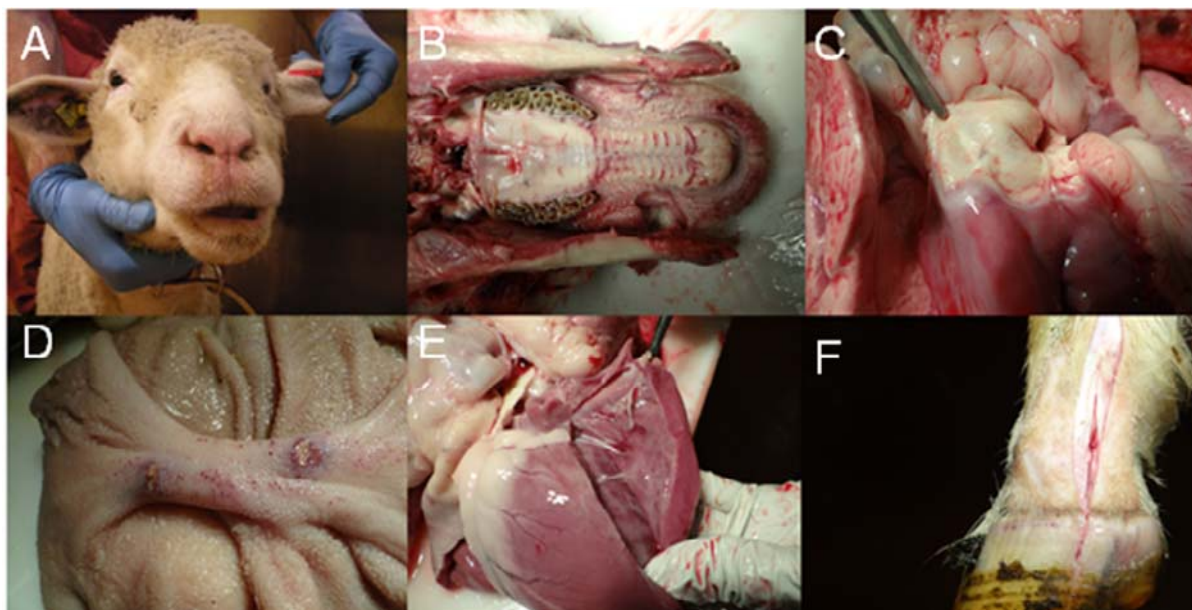
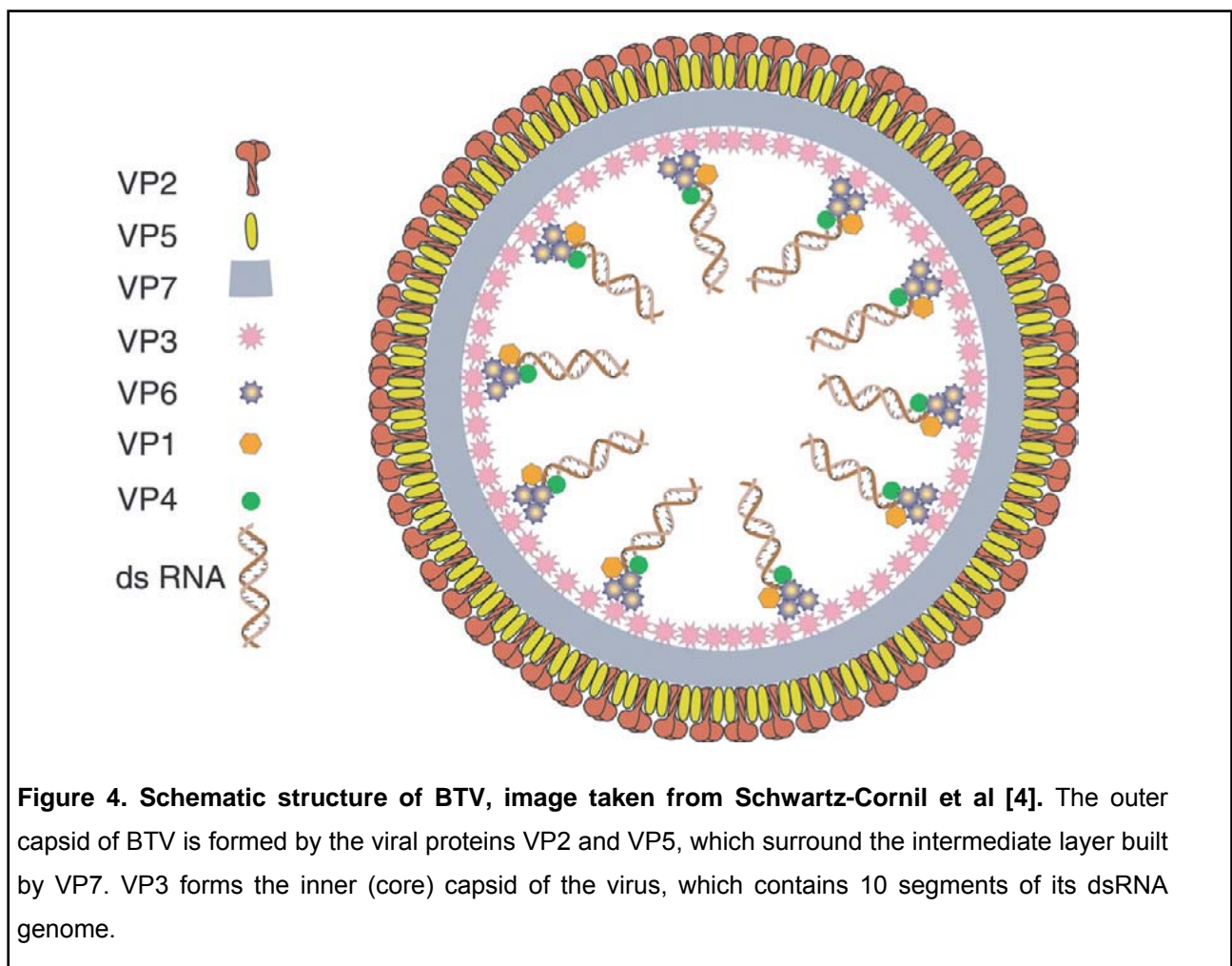


Figure 3. Acute disease and pathological findings in Bluetongue virus-infected sheep. Bluetongue disease gross lesions in BTV-8 infected sheep at 8 dpi; the animal displays severe facial oedema, nasal discharge and salivation (A). Pathological findings at 14 dpi (B) hard palate with linear hemorrhages following the tips of the rugae, (C) haemorrhagic focus at the base of the pulmonary artery, (D) several hemorrhagic foci covered with fibronecrotic material at the pilae ruminis, (E) hemorrhages at the papillary muscle of the heart, (F) petechiae of the coronary band.

Structure and replication cycle

The BTV genome consists of 10 segments of dsRNA, which encode 7 structural (VP1 - VP7) and 5 non-structural proteins (NS1 – NS4, NS3a) [19, 20]. The non-enveloped icosahedral virion is composed of three concentric protein layers that surround the inner core (**Figure 4**). The core includes the viral RNA, VP1 (RNA polymerase), VP4 (capping enzyme), and VP6 (helicase) [21, 22]. The inner capsid layer consists of a heterodimer of VP3 that forms a scaffold for VP7. The outer capsid is formed by the viral proteins VP2 and VP5, which are responsible for receptor binding in the mammalian host, hemagglutination, and membrane penetration, respectively [23, 24] (**Figure 5**). The large (110 kDa) attachment protein VP2 induces virus-neutralizing antibodies [25]. However, VP2 is highly variable and

currently 26 different BTV serotypes can be distinguished by antibodies that show little or no cross-neutralizing activity [4]. Binding of VP5 to VP2 leads to a VP2 conformational change, which may affect the epitopes of neutralizing antibodies [26, 27]. VP5, which is significantly more conserved than VP2, has been shown to be a membrane penetration protein and facilitates the release of the core from the endosome into the cytoplasm [28]. All other structural and non-structural proteins are relatively conserved among different BTV serotypes. As a consequence, most ELISAs for detection of group-specific -BTV antibodies employ the VP7 antigen [29].



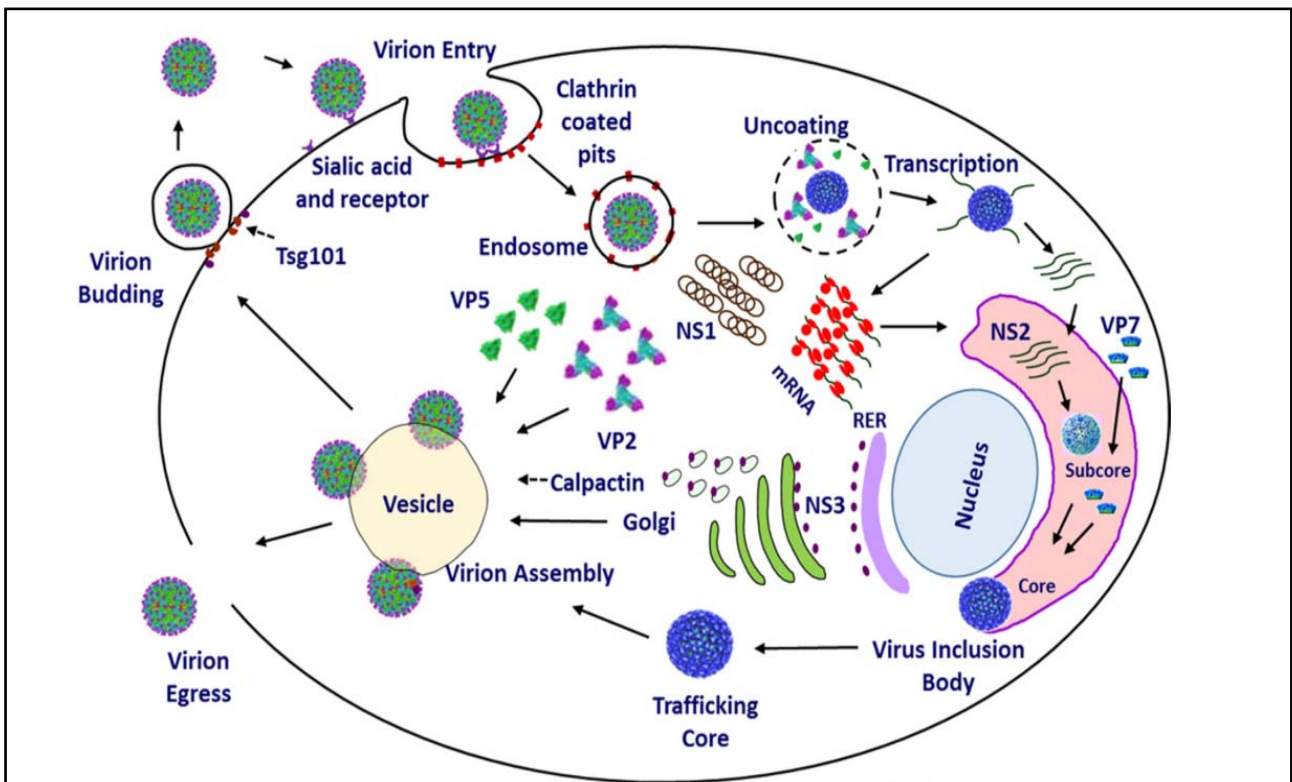


Figure 5. Overview of the replication cycle, adapted from Patel and Roy [30]. Following VP2-mediated attachment to sialic acid-containing receptors, clathrin-mediated endocytosis takes place. Acidification of the endosome followed by VP5-mediated membrane permeabilisation leads to release of the core particle into the cytosol of the host cell. After transcription and translation of the viral proteins, core particles are assembled in viral inclusion bodies and virions are assembled. They then egress via budding or host cell lysis.

Vaccine strategies

Currently available commercial vaccines against BTV are either attenuated or inactivated. Live-attenuated (MLV) vaccines have been used to control BT in sheep in Southern Africa, the United States and Israel as they induce solid immunity and can be produced relatively cheaply [31]. However, potential complications of MLV vaccines include transient viremia after vaccination – possibly leading to further distribution by vector midges - making them unsuitable candidates for use in non-endemic regions [32, 33]. The epizootic of BTV-8 which struck Europe between 2006 and 2008 was controlled by an extensive vaccination campaign using whole

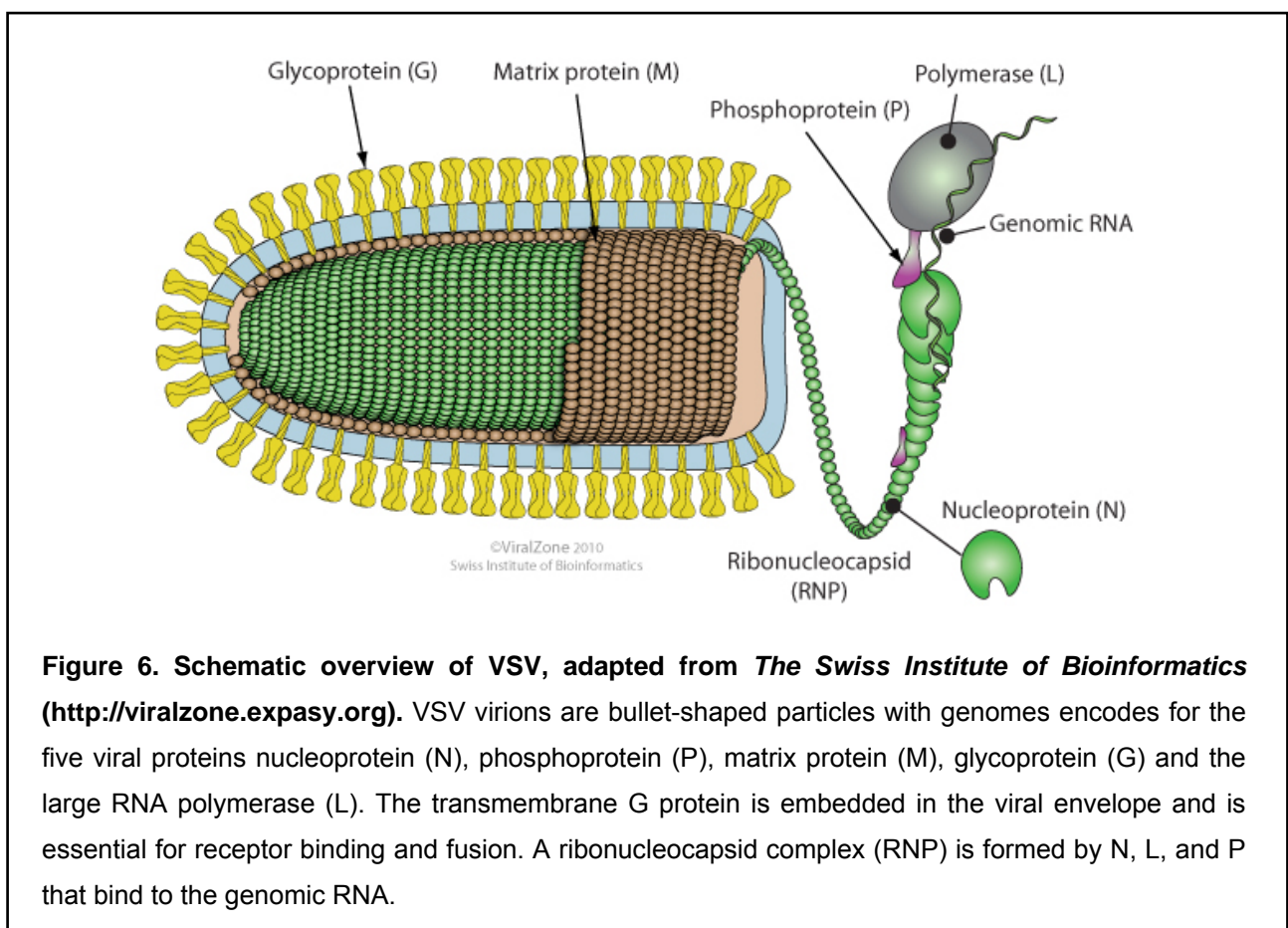
inactivated BTV-8. Although these vaccines induced strong protection against BTV-8 infection and disease, they did not allow simple serological discrimination of infected from vaccinated animals (DIVA). Furthermore, vaccine production required the production of large amounts of infectious virus in cell culture, proper virus inactivation, and formulation of the antigen with adjuvant, all of which delayed production and added to the cost of producing the vaccine. Importantly, inactivated virus vaccines may not be suitable for the control of all serotypes of BTV as, for example, serotype 25 cannot yet be propagated in cell culture [34].

Much effort has been devoted to the development of alternative vaccine candidates, to overcome the drawbacks of conventional vaccines. Virus-like particles were produced using recombinant baculovirus-encoded BTV proteins that were expressed using baculovirus expression vectors [35, 36]. They proved to be promising candidates for the production of multi-serotype vaccines, but also caused concerns regarding potential replication in insect vectors in the field [37]. Other recombinant viruses encoding different BTV-antigens such as vaccinia viruses [38], canarypox viruses [39], capripox viruses [40] and herpes viruses [41] have also been proposed. These studies already revealed the importance of the two components of the outer BTV shell, VP2 and VP5, for protection [42]

INTRODUCTION TO VSV REPLICONS

Vesicular stomatitis virus (VSV) structure and replication

VSV is a non-segmented negative-strand RNA virus of the family *Rhabdoviridae*, and is known to trigger a robust humoral immune response in many different host species [43]. Virions are bullet-shaped particles with genomes which encode for the five viral proteins, namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L), resulting in a total size of approximately 11.2 kb. (**Figure 6**). The virus is characterized by a broad cell tropism with propagation to high titres and cytopathogenicity (**Figure 7**).



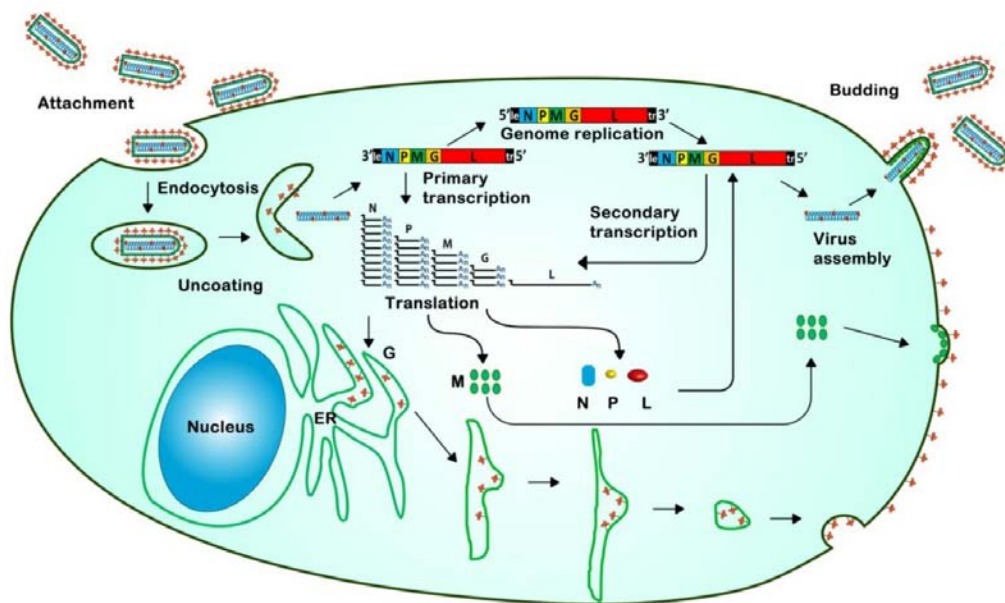


Figure 7. Replication cycle of VSV, adapted from Jianrong Li and Yu Zhang [44] VSV attachment is mediated by the outermost G to host cell receptors, which leads to a clathrin-mediated endocytosis. The ribonucleoprotein complex (RNP) is released into the cytoplasm, where transcription and replication takes place. Finally, the virus assembles and buds at the plasma membrane, releasing new virions.

Vesicular stomatitis virus replicons in experimental vaccines

At first, VSV with a deleted G gene, resulting in a propagation-incompetent VSVΔG, was used for pseudotyping experiments with heterologous glycoproteins [45]. Later, recombinant VSV vectors have been frequently used for experimental vaccination of mammals against a number of different pathogens such as SARS [46], HIV-1 [47], human papilloma virus [48], hepatitis C virus [49], and respiratory syncytial virus [50]. A modified VSV vector in which the gene expressing VSV glycoprotein (G) was replaced by the influenza HA gene was shown to protect chicken from challenge infection with highly pathogenic avian influenza viruses [51, 52]. Due to the deletion of the G protein gene, the vector was restricted to a single round of infection contributing to the excellent biosafety profile of this viral vector. These single-cycle

virus vector vaccines were effective even though adjuvants were not employed [51, 52].

Generation of VSV replicon particles expressing foreign antigens

To generate replication-incompetent VSV replicon particles (VRPs) expressing high levels of foreign antigens, plasmids carrying the genes of interest instead of the viral glycoprotein G have to be designed, employing a reverse genetics system and helper cell lines, which are able to provide the lacking G protein *in trans*. (**Figure 8**) [53].

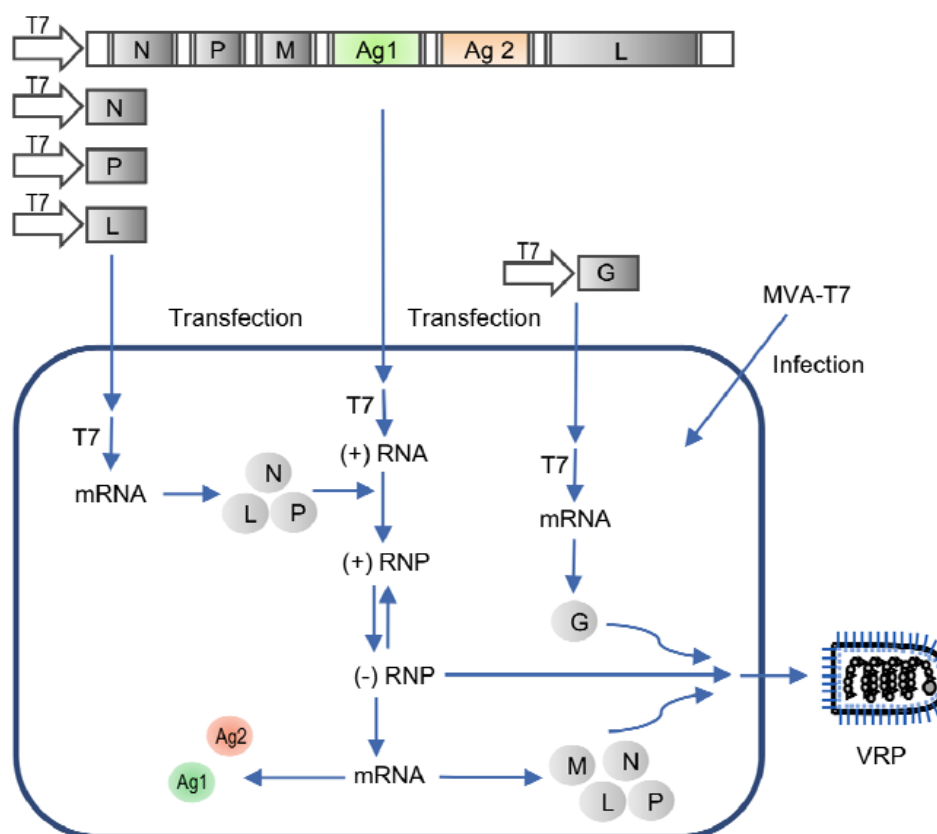


Figure 8. Generation of virus replicon particles encoding foreign antigens based on VSV, illustration taken from Zimmer [54]. The VSV the replicon RNA encodes for the N, P and L proteins, which constitute the viral RNA polymerase complex, the matrix protein M, and two heterologous antigens (Ag1 and Ag2). The replicon RNA lacks the gene encoding for the viral envelope glycoprotein G.

AIM OF THE PROJECT

Outbreaks of BT disease amongst domestic and wild ruminants can cause considerable economic loss that reflect high morbidity and mortality as well as indirect losses because of trade restrictions [10]. In the European outbreak of BTV-8 between 2006 and 2008, an inactivated vaccine was successfully employed. However, a serological discrimination between infected and vaccinated animals was not possible.

In this project, we aimed on overcoming these hurdles using propagation-incompetent VSV Δ G vectors as a new vaccine platform against BTV, which should be safe, efficacious and conferred the DIVA principle.

First, we generated VSV-virus replicon particles expressing different combinations of vaccine candidates and studied the expression of recombinant BTV antigens. Next, we assessed the immune response in a small number of sheep towards VSV VRPs compared to the inactivated vaccine. Immunized sheep were then infected with pathogenic BTV-8. Next, we immunized a small number of sheep to test the VRP's general suitability, using different combinations of vaccine candidates and comparing results to animals immunized with the inactivated vaccine before and after infection with infectious BTV-8 and monitored for viremia, clinical symptoms, and antibody production. Taking the results from these first experiments into account, we finally selected propagation-incompetent VSV Δ G vectors expressing the BTV-8 outer capsid proteins VP2 or VP5, or a combination of both, and performed a blind vaccine/challenge study with 24 sheep. The DIVA principle was validated using a commercially available VP7-based competitive ELISA.

RESULTS

Unpublished Results

Generation of recombinant VSV replicons expressing BTV antigens

The vaccine vector system is based on a vesicular stomatitis virus (VSV) deletion mutant that lacks the essential envelope glycoprotein G in the fourth transcription unit and expresses the enhanced green fluorescent protein at position 5 (VSV*ΔG). By inserting different BTV-8 antigens of interest VP2, VP5, VP3, VP7 and the non-structural protein NS1 and NS3 into the fourth transcription unit, we were able to design six different single antigen expression vectors (**Figure 9**). Three vectors, expressing two BTV-8 genes from the fourth and fifth transcription unit were also designed. Recombinant VSV*ΔG served as a control vector as it did not express any BTV antigen. All recombinant vectors were propagated on helper cell lines providing the VSV glycoprotein G *in trans*, which yielded infectious virus replicon particles (VPRs) of approximately 10^8 infectious units per ml of cell culture supernatant.

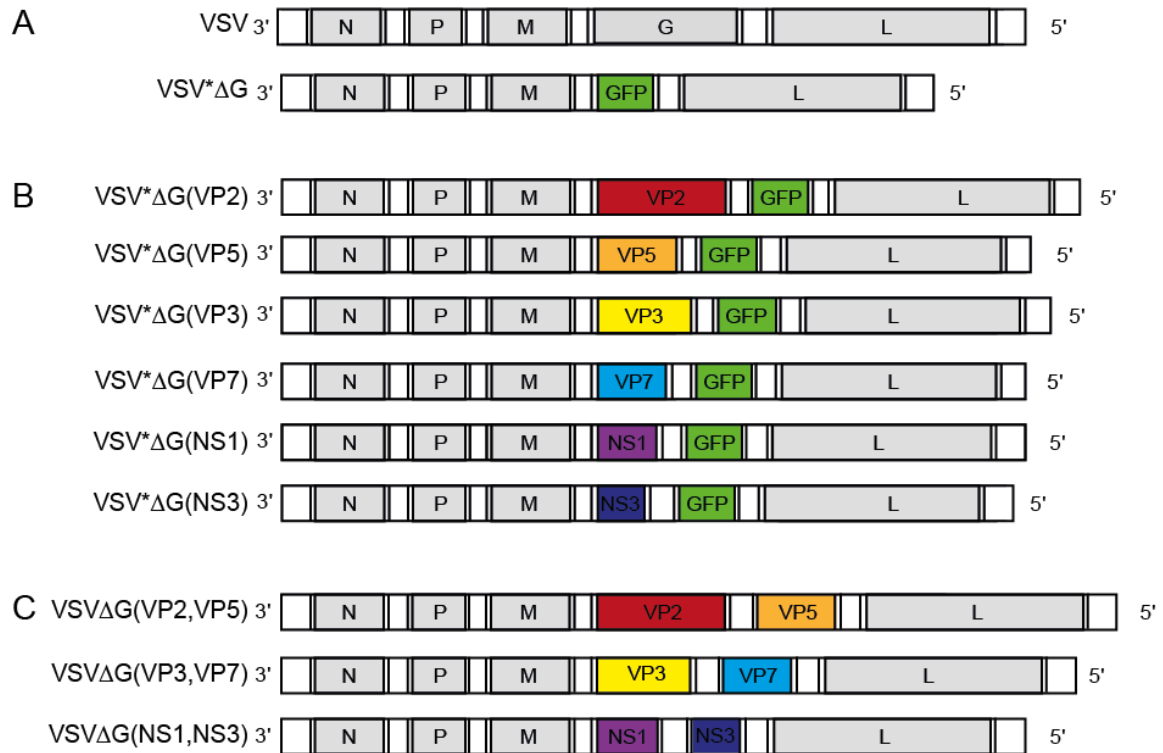


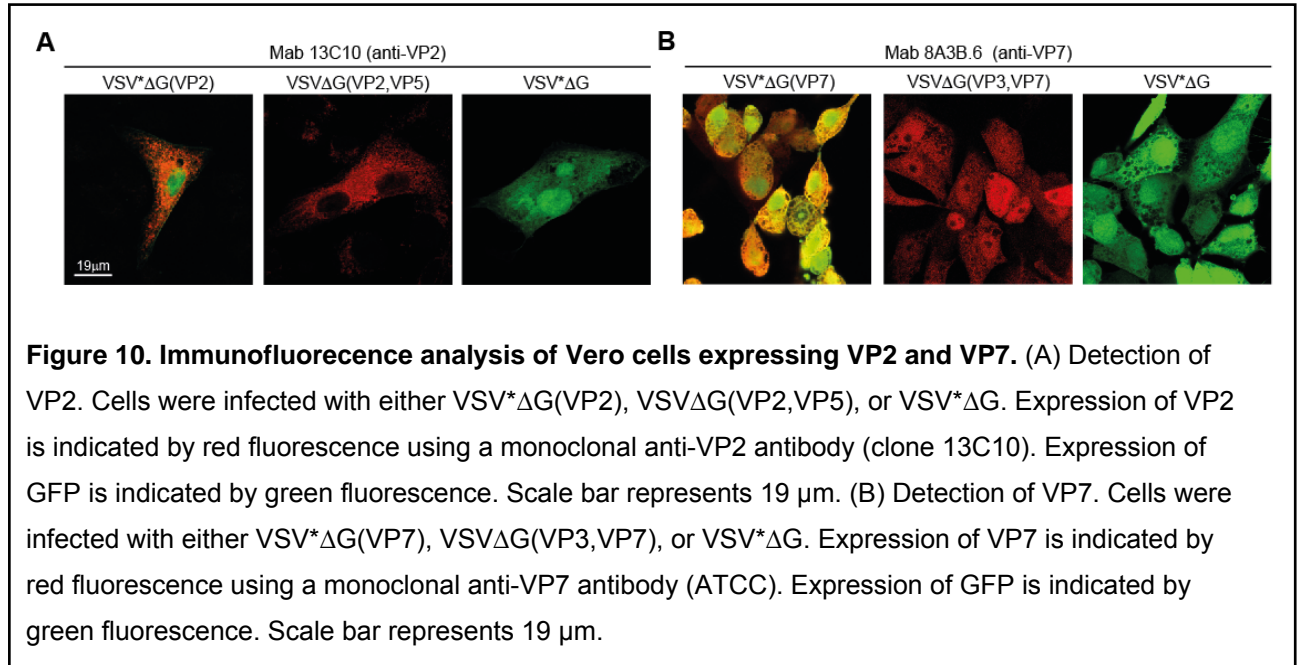
Figure 9. Genome maps of recombinant VSV. (A) VSV encodes for nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L). In the VSV*ΔG, the glycoprotein (G) has been replaced by the GFP gene (denoted by *). (B) The vectors VSV*ΔG(VP2), VSV*ΔG(VP5), VSV*ΔG(VP3), VSV*ΔG(VP7), VSV*ΔG(NS1), and VSV*ΔG(NS3) express individual antigens of BTV-8 from the fourth transcription unit while GFP (*) is expressed from an additional transcription unit downstream of the BTV antigen. (C) Double expression vectors VSVΔG(VP2,VP5), VSVΔG(VP3,VP7), and VSVΔG(NS1, NS3) express two BTV-8 antigens from the fourth and fifth position.

Characterization of antigen expression *in vitro*

In order to study the expression of BTV antigens VP2 and VP7, we performed indirect immunofluorescence analysis (IFA) with Vero cells infected with VRPs using an MOI of 1.

The BTV-8 VP2-specific monoclonal antibody reacted with cells infected with VSV*ΔG(VP2) and VSVΔG(VP2, VP5), whereas cells infected with VSV*ΔG were not recognized (**Figure 10A**). Expression of VP7 was detected by a VP7-specific

monoclonal antibody in cells infected with VSV*ΔG(VP7) and VSVΔG(VP3,VP7), whereas cells infected with VSV*ΔG were not recognized (**Figure 10B**).



Because specific monoclonal antibody were not available, expression of VP5 and NS1 was confirmed using modified antigens, containing a triple FLAG epitope at the C-terminus. The monoclonal anti-FLAG antibody reacted specifically with Vero cells infected with VSV*ΔG(VP5-FLAG), VSVΔG(VP2,VP5-FLAG) and VSV*ΔG(NS1-FLAG) but not with VSV*ΔG(VP5)-, VSV*ΔG(NS1)-, or VSV*ΔG-infected cells (**Figure 11**).

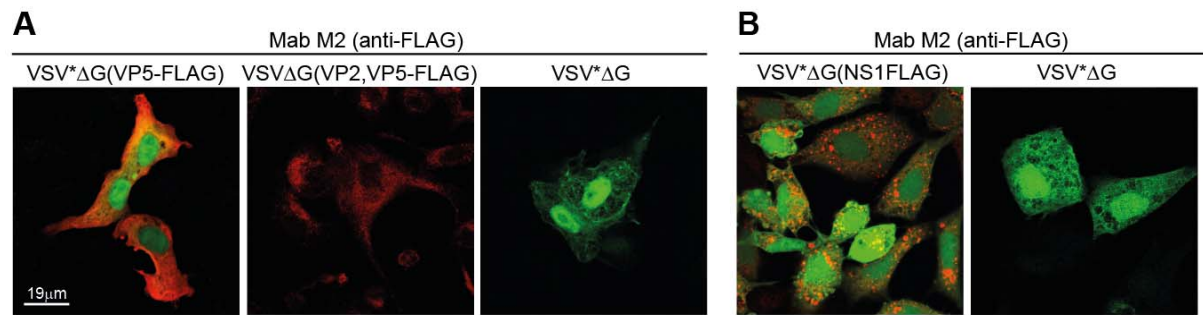


Figure 11. Immunofluorescence analysis of Vero cells expressing VP5 and NS1. (A) Cells were infected with either VSV*ΔG(VP5-FLAG), VSVΔG(VP2,VP5-FLAG), or VSV*ΔG. Expression of VP5 is indicated by red fluorescence using a monoclonal anti-FLAG antibody. (B) Cells infected with either VSV*ΔG(NS1-FLAG), or VSV*ΔG. Expression of NS1 is indicated by red fluorescence using a monoclonal anti-FLAG antibody.

For the remaining BTV antigens VP3 and NS3, monoclonal antibodies were not available. In order to obtain evidence for expression of these antigens, chickens were immunized with recombinant VSV replicons. Immune sera were then tested on Vero cells 24 h post infection with BTV-8 (**Figure 12**). All sera were found to react specifically with the infected cells.

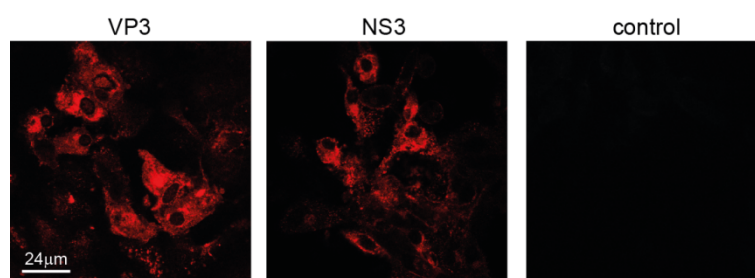


Figure 12. Immunofluorescence analysis of BTV-8 infected Vero cells stained with anti-VP3 and anti-NS3 immune sera. Sera obtained from chicken immunized with VSV-VRPs react with BTV-8 infected cells (red fluorescence). Scale bar represents 24 μm.

Taken together, these data show that infection of Vero cells with virus replicon particles (VRP) readily led to expression of BTV-8 antigens VP2, VP5, VP7 and NS1.

Evidence of expression of BTV-8 antigens VP3 and NS3 was obtained indirectly, since sera of immunized chickens reacted with BTV-8 infected cells. However, infectious VSV was not produced by the cells due to the lack of VSV G protein expression. Thus, we refer to the recombinant VSV vectors as virus replicon particles (VRP).

Analysis of antibody responses in VRP-immunized sheep

To evaluate the immunogenicity of recombinant VRPs in a natural BTV host, nine seronegative sheep were immunized three times at 3-week intervals. Three animals were immunized with cell culture supernatant containing at least 1×10^8 infectious units of each of the VRPs VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3). Two animals were immunized with the VRPs VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) expressing four antigens in total. Four animals received VSV* Δ G as control. Furthermore, five animals that had been immunized with inactivated BTV-8 vaccine (BTVpur) two years ahead of the experiment were included in our analysis. No adverse side effects due to vaccination with VRPs were observed.

Three weeks after the third immunization, sera were prepared from sheep and analyzed for the presence of BTV-specific antibodies by immunofluorescence. All sera of sheep immunized with VRPs containing BTV antigens and BTVpur reacted specifically with BTV-8 infected cells, indicating that antibodies had been produced (**Figure 13**). In contrast, sera from VSV* Δ G-immunized sheep did not react.

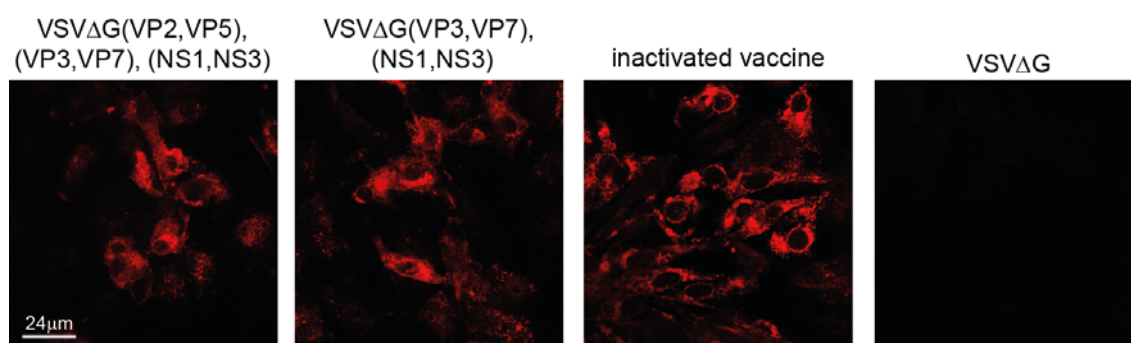


Figure 13. Immunofluorescence analysis of BTV-8 infected Vero cells at 24 hpi with BTV-8. Sera obtained from sheep after triple immunization with VSV-VRPs react with BTV-8 infected cells (red fluorescence). Scale bar represents 24 μ m.

Sera were tested for virus neutralizing activity against BTV-8. Sera of sheep before immunization with VSV-VRPs did not show any neutralizing activity confirming that the animals had no pre-existing immunity to BTV-8 (**Figure 14**). In contrast, sera of animals vaccinated with the commercially available inactivated vaccine (BTVpur) had neutralizing activity. Sera collected 3 weeks after the first immunization showed some neutralizing activity at the highest serum concentration (1:4) tested. However, 3 weeks after the third immunization with the vaccine cocktail VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7), and VSV Δ G(NS1,NS3), all animals in the group developed high neutralizing antibody titres against BTV-8. The same sera did not neutralize BTV-1 or the VSV vector (data not shown). Sheep immunized with either VSV* Δ G or VSV Δ G(VP3,VP7) in combination with VSV Δ G(NS1,NS3) did not develop BTV-8 neutralizing antibodies. Taken together, these data demonstrate that the recombinant VRPs induced antibodies with neutralizing activity *in vitro*, when VP2 and VP5 were expressed from the VSV vector.

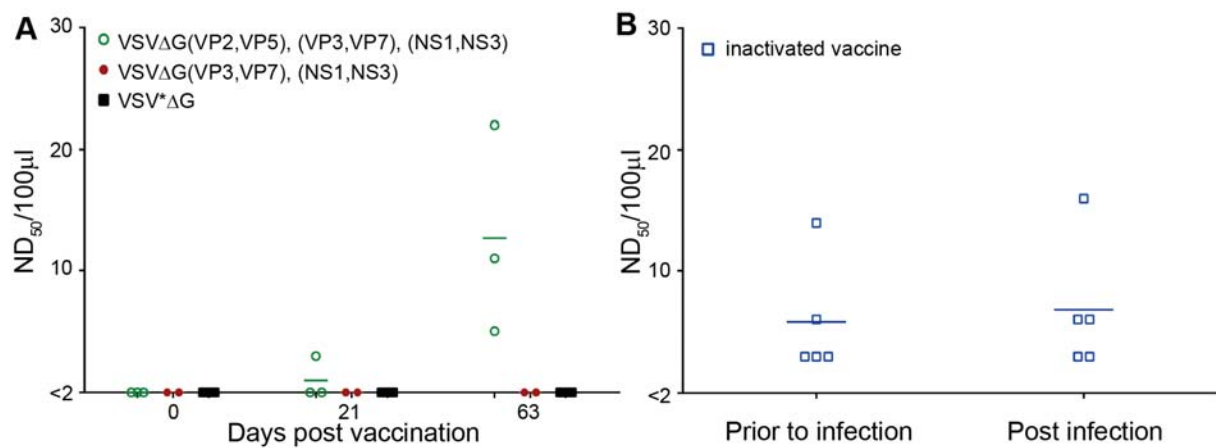
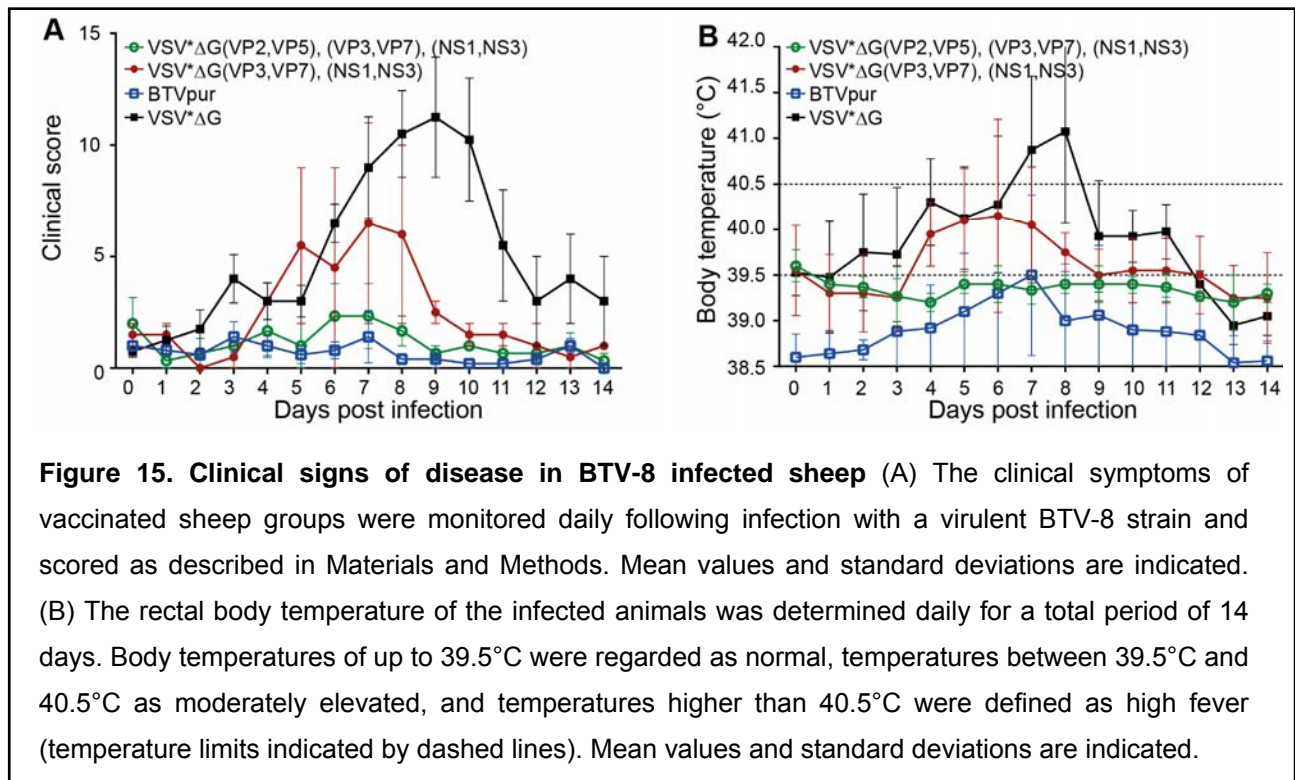


Figure 14. Neutralizing antibodies in sera of immunized sheep. Detection of BTV-8 neutralizing serum antibodies. (A) Sera were prepared from VRP-immunized sheep at day 0 (first vaccination), day 21 (second vaccination) and at day 63 (three weeks after the second immunization, prior to challenge infection). (B) Sera were prepared from sheep, immunized with inactivated BTV-8 vaccine (BTVpur) before and (14days) after infection with BTV-8. The infection or protection of cells was estimated 3 to 5 dpi according to the development of CPE. Neutralizing titres were calculated and expressed as ND₅₀/100 µl. Values equal to or lower than 2 were regarded negative.

VRPs expressing a combination of VP2 and VP5 antigens protect sheep from challenge with pathogenic BTV-8

To evaluate the capacity of recombinant VRP vaccines to provide protection against pathogenic BTV-8, sheep were inoculated i.v. with erythrocyte suspension containing BTV-8 (C_q value of 18) 3 weeks after the second booster immunization. Sheep immunized with VSV*ΔG showed characteristic signs of bluetongue disease beginning at 5 dpi (**Figure 15A**). Typical symptoms were facial edema, nasal discharge, and lethargy. Two animals from this group were euthanized for humane reasons at day 11 post infection, as they developed severe lung edema and respiratory distress. All animals of this groups developed high fever, which peaked at day 8 after infection (**Figure 15B**). Animals immunized with the combination of VSVΔG(VP3,VP7) and VSVΔG(NS1,NS3) developed milder clinical symptoms (**Figure 15A**) and recovered from moderately elevated inner body temperature

(**Figure 15B**), sooner. Animals immunized with either the combination of VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) or the inactivated vaccine BTVpur did not develop BTV-specific clinical signs of disease (**Figure 15A**) and had normal rectal temperature ($< 39.5^{\circ}\text{C}$) throughout the experiment (**Figure 15B**).



The capacity of the vaccine-induced immune response to suppress viremia was evaluated by determining viral RNA loads in blood by quantitative RT-PCR (**Figure 16**). Animals immunized with either VSV* Δ G or combined VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) had high levels of viral RNA in blood, starting from day 2 post challenge and lasting until the end of the experiment at day 14. In contrast, BTV RNA was not detected in blood of sheep immunized with either the vaccine cocktail VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) or the inactivated

vaccine (BTVpur). Thus, recombinant VRPs expressing VP2 and VP5 induced protective immunity in sheep that prevented both viremia and bluetongue disease.

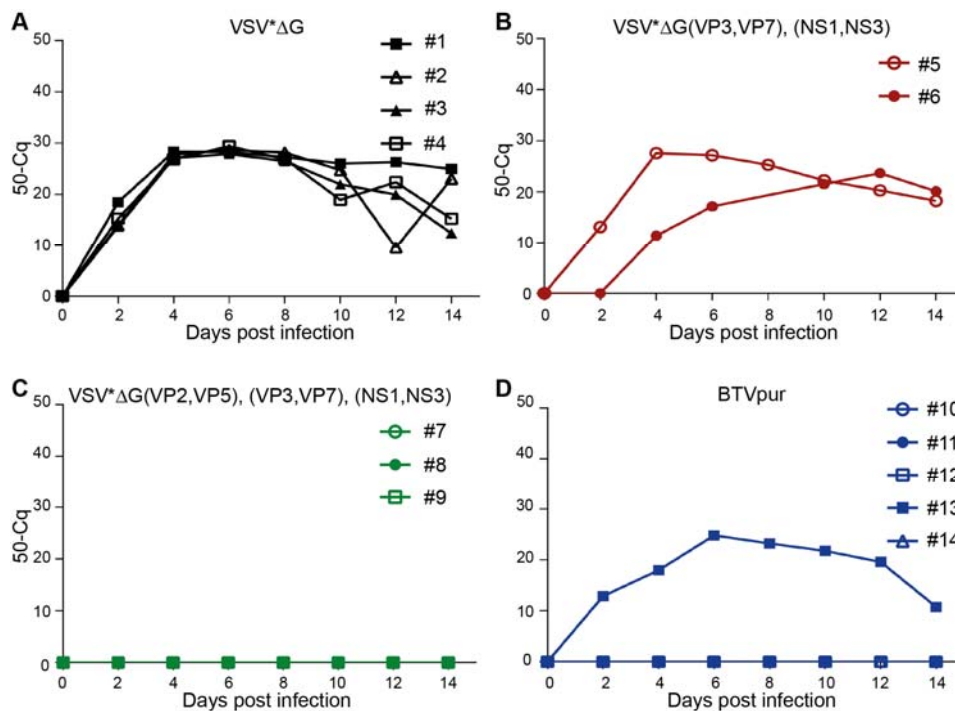


Figure 16. Determination of viral RNA loads in blood of BTV-8 challenged sheep. Blood was collected from animals of the indicated vaccine groups every second day after infection with BTV-8. Total RNA was extracted from whole blood samples and RT-qPCR was performed to determine viral RNA loads. The quantification cycles (C_q) for detection of fluorescence signals were expressed as 50- C_q for all 14 animals (numbered #1 to #14).



RESEARCH

Open Access

Vesicular stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus serotype 8 induces complete protection of sheep against challenge infection

Stefanie Kochinger, Nathalie Renevey, Martin A Hofmann and Gert Zimmer*

Abstract

Bluetongue virus (BTV) is an arthropod-borne pathogen that causes an often fatal, hemorrhagic disease in ruminants. Different BTV serotypes occur throughout many temperate and tropical regions of the world. In 2006, BTV serotype 8 (BTV-8) emerged in Central and Northern Europe for the first time. Although this outbreak was eventually controlled using inactivated virus vaccines, the epidemic caused significant economic losses not only from the disease in livestock but also from trade restrictions. To date, BTV vaccines that allow simple serological discrimination of infected and vaccinated animals (DIVA) have not been approved for use in livestock. In this study, we generated recombinant RNA replicon particles based on single-cycle vesicular stomatitis virus (VSV) vectors. Immunization of sheep with infectious VSV replicon particles expressing the outer capsid VP2 protein of BTV-8 resulted in induction of BTV-8 serotype-specific neutralizing antibodies. After challenge with a virulent BTV-8 strain, the vaccinated animals neither developed signs of disease nor showed viremia. In contrast, immunization of sheep with recombinant VP5 - the second outer capsid protein of BTV - did not confer protection. Discrimination of infected from vaccinated animals was readily achieved using an ELISA for detection of antibodies against the VP7 antigen. These data indicate that VSV replicon particles potentially represent a safe and efficacious vaccine platform with which to control future outbreaks by BTV-8 or other serotypes, especially in previously non-endemic regions where discrimination between vaccinated and infected animals is crucial.

Introduction

Bluetongue is a hemorrhagic disease of ruminants that is caused by bluetongue virus (BTV), a member of the genus *Orbivirus* within the family *Reoviridae* [1-3]. BTV is transmitted to livestock by blood-feeding *Culicoides* midges. In cattle, goats, and wild ruminants, BTV infection is typically asymptomatic despite prolonged viremia. These host species represent a potential reservoir for unnoticed dissemination of BTV in ruminant populations. In sheep, however, BTV infection often results in an acute disease with associated high morbidity and mortality, depending on the virulence of the virus and the sheep breed affected [4]. Typical symptoms of bluetongue in sheep include high fever, tissue edema, hemorrhages, and necrosis of the

upper gastrointestinal tract as well as of skeletal and cardiac musculature. Certain strains of BTV, notably the northern European strain of BTV-8, can cross the placental barrier, leading to infection of the developing fetus [5]. Hence, infection of pregnant animals with certain strains of the virus are frequently associated with abortions and malformations of offspring [6-8].

The BTV genome consists of 10 segments of dsRNA, which encode 7 structural (VP1 - VP7) and 5 non-structural proteins (NS1 - NS4, NS3a) [9]. The non-enveloped icosahedral virion particle contains an inner core which is formed by the viral RNA and the viral proteins VP1 (RNA polymerase), VP4 (capping enzyme), and VP6 (helicase) [10,11]. The inner core is surrounded by 3 concentric protein layers. The inner capsid layer consists of 120 copies of VP3 arranged as 60 dimers that form a scaffold for VP7. The outer capsid is composed of the viral proteins VP2

* Correspondence: gert.zimmer@ivl.admin.ch
 Institute of Virology and Immunology (IVI), Sensemattstrasse 293, CH-3147
 Mitholz, Switzerland



© 2014 Kochinger et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.

and VP5, which are responsible for receptor binding, hemagglutination, and membrane penetration, respectively [12,13]. The large (110 kDa) attachment protein VP2 induces virus-neutralizing antibodies [14]. However, VP2 is highly variable and currently 26 different BTV serotypes can be distinguished by antibodies that show little or no cross-neutralizing activity [3]. Binding of VP5 to VP2 leads to a VP2 conformational change, which may affect recognition of epitopes by neutralizing antibodies [15,16]. All other structural and non-structural proteins are relatively conserved among different BTV serotypes. Therefore, most ELISAs for pan-BTV antibody detection employ the VP7 antigen [17].

A novel strain of BTV serotype 8 (BTV-8), which had not been detected in Europe before 2006, emerged as an epidemic wave in Western and Northern Europe [3,18,19]. This outbreak had a significant economic impact, not only because the disease caused morbidity and mortality in sheep and cattle but also because of restrictions imposed on livestock movement and trade [20]. The epizootic was controlled by a large-scale vaccination campaign using whole inactivated BTV-8. Although this vaccine induced strong protection against BTV-8 infection and disease, it did not allow the simple serological discrimination of infected from vaccinated animals (DIVA). Furthermore, vaccine production required the production of large amounts of infectious virus in cell culture, proper virus inactivation, and formulation of the antigen with adjuvant, all of which delayed production and added to the costs of producing the vaccine. Importantly, inactivated virus vaccines may not be suitable for the control of all serotypes of BTV as, for example, serotype 25 cannot be propagated in cell culture [21]. To overcome these hurdles, our goal was to develop and evaluate a generic vector vaccine in sheep, one that would comply with the DIVA principle. Although similar strategies using recombinant poxviruses (vaccinia, goatpox, and canarypox viruses), herpesviruses, and virus-like particles have been proven effective, we elected to evaluate the vesicular stomatitis virus (VSV) replicon system [22].

VSV is a non-segmented negative-strand RNA virus that is known to trigger a robust humoral immune response in many different host species [23]. Recombinant VSV vectors have been employed for experimental vaccination of mammals against a number of different pathogens such as human papilloma virus, hepatitis C virus, or influenza A virus [24]. A modified VSV vector in which the VSV glycoprotein (G) gene was replaced by the influenza A virus HA gene was shown to protect chickens from challenge infection with highly pathogenic avian influenza viruses [25,26]. This vector was propagated to high titers on helper cells providing the VSV G protein *in trans* and was able to infect a broad spectrum of different cell types. Due to the autonomous replication of the viral RNA, high-level

expression of recombinant antigens was achieved. However, due to the deletion of the VSV G protein gene, VSVΔG vectors are restricted to a single round of infection, contributing to their excellent biosafety profile. These novel vector vaccines were effective even though adjuvants were not employed [25,26].

In this study, propagation-incompetent VSVΔG vectors expressing the BTV-8 outer capsid proteins VP2 or VP5, or a combination of both, were generated. The expression of recombinant BTV antigens was studied and the immune response in sheep assessed. Immunized sheep were then infected with virulent BTV-8 and monitored for viremia, clinical symptoms, and antibody production. The DIVA principle was assessed using a commercially available VP7-based competitive ELISA.

Materials and methods

Cells

BHK-21 cells were obtained from the German Cell Culture Collection (DSZM, Braunschweig, Germany) and grown in Earle's minimal essential medium (MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Biowest Nuaillé, France). BHK-G43, a transgenic BHK-21 cell clone expressing the VSV G protein in a regulated manner, was maintained as described previously [27]. Vero cells (C1008) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Glasgow's minimal essential medium (GMEM; Life Technologies) supplemented with 5% FBS.

Viruses

A German BTV-8 isolate (from 2008) was kindly provided by Bernd Hoffmann (FLI, Riems, Germany). A French isolate of BTV-1 was obtained from The Pirbright Institute (Pirbright, UK). Virus stocks were titrated on Vero cells. Virus infection was monitored taking advantage of the cytopathic effect apparent at day 5 post infection (pi). Infectious virus titres were calculated according to the Spearman-Kärber method and expressed as 50% tissue culture infectious doses per mL (TCID₅₀/mL).

In order to obtain virulent BTV-8 for challenge experiments, two adult BTV-seronegative Poll Dorset sheep were inoculated intravenously using erythrocytes prepared from a BTV-8 viremic calf. This virus was originally isolated in 2008 in Gummersbach, Germany. Following infection, blood was collected at daily intervals and analyzed for the presence of viral RNA by RT-qPCR [28]. The sheep were euthanized at day 5 pi, when a quantification cycle (C_q) value of 18 was determined in blood by RT-qPCR. Blood was collected from the animal, erythrocytes were washed and suspended in PBS, and frozen at -70 °C.

Construction of recombinant VRPs

Codon-optimized cDNA of VP2 (GenBank accession number AM498052) and VP5 (GenBank accession number AM498056) of the BTV-8 strain NET2006/04 was synthesized by GenScript (Piscataway, NJ, USA). For generation of recombinant VSV, the BTV-8 genes were inserted into the plasmid pVSV* using *MluI* and *BstEII* restriction sites upstream and downstream of the fourth transcription unit, thereby replacing the VSV G gene [25]. The resulting plasmids were designated pVSV*ΔG(VP2) and pVSV*ΔG(VP5), respectively. For generation of a dual antigen expression vector, the VP5 cDNA was inserted into pVSV*ΔG(VP2) using *XhoI* and *NheI* restriction sites located upstream and downstream of the fifth transcription unit, thereby replacing the green fluorescent protein (GFP) gene. The resulting plasmid was designated pVSVΔG(VP2,VP5). For expression of a modified VP5 containing a short peptide epitope at the C terminus, the VP5 gene without its Stop codon was inserted into the pCMV-3Tag-3 plasmid vector (Agilent Technologies, Inc., Santa Clara, CA, USA) upstream of and in frame with a triple FLAG epitope (DYKDDDDK)-coding region, which was followed by a Stop codon. The VP5-FLAG open reading frame was then amplified by PCR and inserted into either the fourth transcription unit of pVSV*ΔG or the fifth transcription unit of pVSV*ΔG(VP2). The resulting plasmids were designated pVSV*ΔG(VP5-FLAG) and pVSVΔG(VP2,VP5-FLAG), respectively. VSV replicon particles (VRPs) were generated and propagated on BHK-G43 helper cells as described previously [29].

Recombinant VSV*ΔG(VP2), VSV*ΔG(VP5), VSV*ΔG(VP5-FLAG), and VSV*ΔG [25] were titrated on BHK-21 cells taking advantage of the GFP reporter protein. Infectious titers were calculated and expressed as fluorescence-forming units per milliliter (ffu/mL). For detection of VSVΔG(VP2,VP5) and VSV*ΔG(VP2,VP5-Flag), infected cells were fixed with PBS containing 3% paraformaldehyde for 20 min at room temperature, washed with PBS containing 0.1 M (w/v) glycine, and permeabilized with 0.25% (v/v) of Triton-X100. The cells were incubated with rabbit anti-VSV serum and subsequently with a goat anti-rabbit horseradish peroxidase conjugate (dilution 1:500 in PBS; DAKO; Glostrup, Denmark) and finally stained with AEC/H₂O₂ substrate.

Immunofluorescence analysis

Vero cells grown on 12-mm-diameter cover slips were inoculated for 90 min with either VSV*ΔG, VSV*ΔG(VP2), VSV*ΔG(VP5-FLAG), VSVΔG(VP2,VP5) or pVSVΔG(VP2,VP5-FLAG) using a multiplicity of infection (MOI) of 3. At 8 hours pi, the cells were fixed with 3% paraformaldehyde for 20 min, washed with PBS containing 0.1 M (w/v) glycine, and incubated with PBS containing 0.25% (v/v) Triton X-100 for 5 min to permeabilize the plasma

membrane. The cells were incubated with a monoclonal antibody directed against either VP2 (clone 13C10, diluted 1:10; kindly provided by Dr Malte Dauber, FLI Riems, Germany) or a monoclonal antibody directed against the FLAG epitope (clone M2, diluted 1:50; Sigma-Aldrich, Deisenhofen, Germany) and subsequently with an anti-mouse IgG-Alexa 546 conjugate (dilution 1:500; Life Technologies, Carlsbad, CA, USA). Finally, the cells were washed with distilled water and embedded in Mowiol 4–88 (Sigma-Aldrich, Deisenhofen, Germany) mounting medium. The cells were analyzed using a Leica TCS-SL spectral confocal microscope (CFM) and Leica LCS software (Leica Microsystems AG, Glattpburg, Switzerland).

To analyze sera from immunized sheep for the presence of virus-specific antibodies, Vero cells were grown on 12-mm-diameter cover slips, infected with BTV-8 (MOI of 1), and incubated for 48 h with medium containing 0.9% (w/v) methylcellulose. The cells were fixed, permeabilized, and incubated with serum (diluted 1:100 in PBS with 1% BSA) from immunized sheep and subsequently with anti-sheep IgG-Alexa 488 conjugate (dilution 1:500 in PBS; Life Technologies, Carlsbad, CA, USA). Fluorescence microscopy was performed using an Axio Observer Z1 inverted microscope (Zeiss, Jena, Germany).

Animal experiments

Animal trials were performed in compliance with the Swiss Animal Protection Law and approved by the Animal Welfare Committee of the Canton of Berne (authorization number 112/12). Twenty-four white Swiss White Alp sheep (12 to 24 months old), that were tested seronegative for BTV by ELISA (see below), were immunized intramuscularly with 2.5 mL of cell culture supernatant containing either VSV*ΔG, VSV*ΔG(VP2), VSV*ΔG(VP5), or VSVΔG(VP2,VP5), respectively. Three weeks (day 21) after the primary immunization (day 0), the animals received the same vaccine a second time. At day 42, 4 mL of erythrocyte suspension from BTV-8 infected viremic sheep (see above) were injected into the animals intravenously. Following inoculation, the animals were surveyed daily for clinical signs of disease which were scored according to a modified clinical scoring system [30]. All surviving animals were euthanized at day 14 post challenge.

Serological tests

Serum neutralization tests were performed as described previously [31]. Briefly, immune sera (heat-inactivated at 56 °C for 30 min) were serially diluted in FBS-free tissue culture medium and incubated for 1 h at 37 °C with 40 TCID₅₀/50 μL. Vero cells were added and incubated at 37 °C for 5 days. If not neutralized by immune serum, BTV caused a cytopathic effect 3 to 5 days pi. Neutralizing antibody titres were calculated according to the Spearman-Kärber method. For detection of VSV-

neutralizing antibodies, a recombinant VSV (serotype Indiana) expressing GFP was used [32]. A polyclonal anti-VSV serum was used as reference. For detection of VP7-specific serum antibodies, a commercially available competitive ELISA was used according to the manufacturer's protocol (VMRD, Pullman, USA).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from whole blood samples using the Ambion MagMAX blood RNA isolation kit (Applied Biosystems, Foster City, California, USA) and stored at -70°C . For detection of viral RNA, a reverse transcriptase quantitative PCR (RT-qPCR) based on the amplification of segment 10 was performed in duplicates using in vitro-transcribed GFP RNA as internal control [28].

Statistical analysis

Statistical significance ($p < 0.05$) was considered using a two-way ANOVA and multiple comparisons were assessed using GraphPad Prism6 Sidak's post-hoc test.

Results

Generation of recombinant RNA replicon particles expressing BTB antigens

We previously generated a propagation-incompetent VSV vector by replacing the VSV glycoprotein (G) gene in the fourth transcription unit of the viral genome with genes encoding influenza virus antigens [25]. This vector contained an additional transcription unit at position 5, which was used to express the green fluorescent protein (GFP). Based on this vector platform we generated the recombinant viruses VSV Δ G(VP2) and VSV Δ G(VP5) containing the VP2 and VP5 genes of BTB-8 in the fourth transcription unit of the vector (Figure 1A). A vector expressing both antigens, VSV Δ G(VP2,VP5), was constructed by replacing the GFP gene in the fifth position of VSV Δ G(VP2) by the VP5 gene of BTB-8. VSV Δ G(VP5-FLAG) and VSV Δ G(VP2,VP5-FLAG) contained a triple FLAG epitope at the C terminus of VP5 and were generated to ease detection of this antigen. Recombinant VSV Δ G served as control vector as it did not express any BTB antigen [25]. All recombinant viruses were propagated on helper cells providing the VSV glycoprotein G *in trans*,

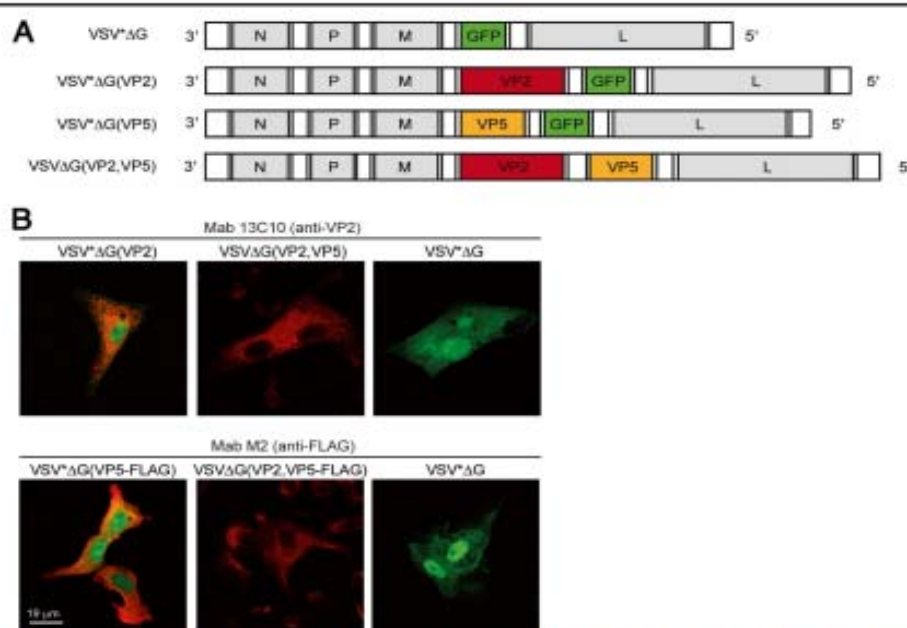


Figure 1 Genome maps and analysis of recombinant protein expression. (A) Genome maps of recombinant VSV. VSV Δ G encodes for nucleoprotein (N), phosphoprotein (P), matrix protein (M), and the large RNA polymerase (L). The VSV glycoprotein (G) has been replaced by the GFP gene (denoted *). VSV Δ G(VP2) and VSV Δ G(VP5) express either VP2 or VP5 antigen of BTB-8 from the fourth transcription unit while GFP is expressed from an additional transcription unit downstream of VP2. VSV Δ G(VP2,VP5) expresses VP2 from the fourth and VP5 from the fifth position. **(B)** Immunofluorescence analysis of Vero cells 8 hpi with either VSV Δ G(VP2), VSV Δ G(VP2,VP5), VSV Δ G(VP5), VSV Δ G(VP5-FLAG), VSV Δ G(VP2,VP5-FLAG), or VSV Δ G. In the upper panel, expression of VP2 is indicated by red fluorescence using a monoclonal anti-VP2 antibody (clone 13C10). In the lower panel, expression of VP5 is indicated by red fluorescence using a monoclonal anti-FLAG (clone M2) antibody. Expression of GFP is indicated by green fluorescence. Scale bar represents 19 μm .

which yielded infectious titres of approximately 10^8 infectious units per mL of cell culture supernatant.

The expression of recombinant BTV-8 proteins was determined by immunofluorescence staining of Vero cells 6 hours pi with recombinant VSV. A BTV-8 VP2-specific monoclonal antibody reacted with cells that were infected with VSV Δ G(VP2) or VSV Δ G(VP2,VP5), whereas cells infected with VSV Δ G(VP5) or VSV Δ G were not recognized (Figure 1B). Since a VP5-specific antibody was not available, expression of VP5 was confirmed using a modified antigen with a triple FLAG epitope at the C terminus.

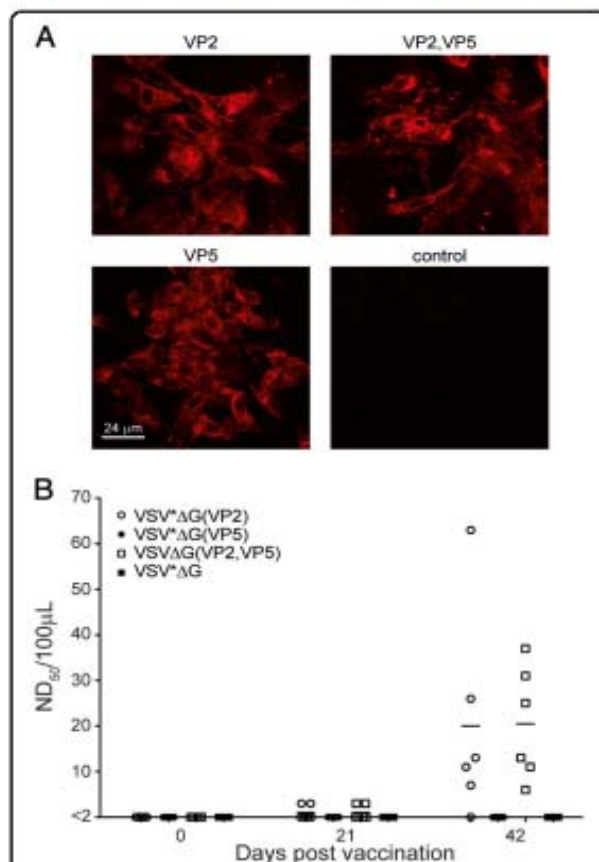


Figure 2 Antibody responses of immunized sheep.

(A) Immunofluorescence analysis of BTV-8 infected cells. Vero cells were infected with an MOI of 1 and further incubated for 24 h at 37 °C. The cells were fixed, permeabilized, and incubated with immune sera from sheep immunized twice with the indicated VRPs. The primary antibodies were detected with anti-sheep IgG-Alexa 546 conjugate (red fluorescence). Scale bar represents 24 µm. (B) Detection of BTV-8 neutralizing serum antibodies. Sera were prepared from VRP-immunized sheep (group size $n = 6$) at day 0 (first vaccination), day 21 (second vaccination), and day 42 (three weeks after the second immunization). Neutralization of virus was estimated 3 to 5 dpi according to the development of CPE. Neutralizing titres were calculated and expressed as ND₅₀/100 µL. Values equal to or lower than 2 were regarded negative.

The monoclonal anti-FLAG antibody reacted specifically with Vero cells infected with VSV Δ G(VP5-FLAG) and VSV Δ G(VP2,VP5-FLAG) but not with VSV Δ G-infected cells (Figure 1B). Taken together, these data show that infection of Vero cells with virus replicon particles readily led to expression of BTV-8 antigens, both VP2 and VP5. However, infectious VSV was not produced by the cells since the VSV G protein was not expressed. Therefore, we refer to the recombinant VSV vectors as virus replicon particles (VRP).

Analysis of antibody responses in VRP-immunized sheep

To evaluate the immunogenicity of recombinant VRPs in a natural BTV host, 12 to 24 month-old Swiss White Alp sheep were divided into 4 groups of 6 animals each and immunized with cell culture supernatant containing at least 1×10^8 infectious units of either VSV Δ G(VP2),

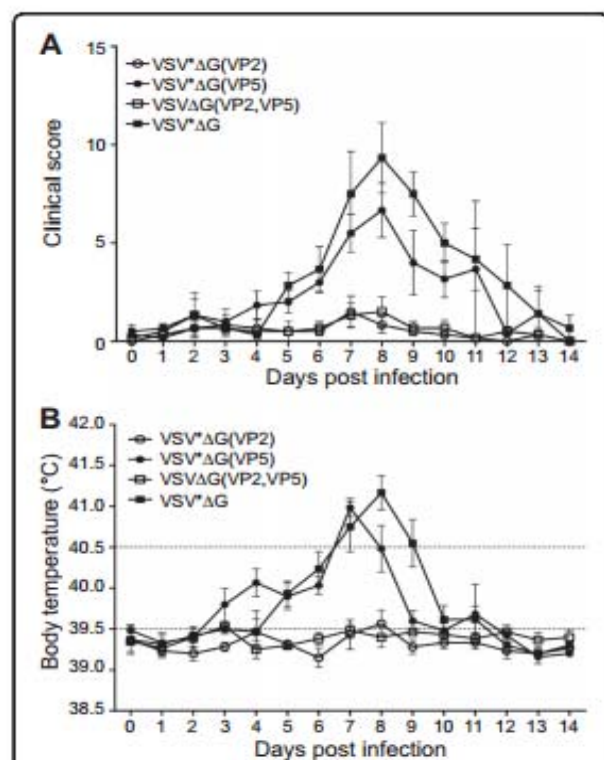


Figure 3 Clinical signs of disease in BTV-8 infected sheep.

(A) The clinical symptoms of vaccinated sheep groups ($n = 6$) were monitored daily following infection with a virulent BTV-8 strain and scored as described in Materials and Methods. Mean values and standard deviations are indicated. (B) The rectal body temperature of the infected animals was determined daily for a total period of 14 days. Body temperatures of up to 39.5 °C were regarded as normal, temperatures between 39.5 °C and 40.5 °C as moderately elevated, and temperatures higher than 40.5 °C were defined as high fever (temperature limits indicated by dashed lines). Mean values and standard deviations are indicated.

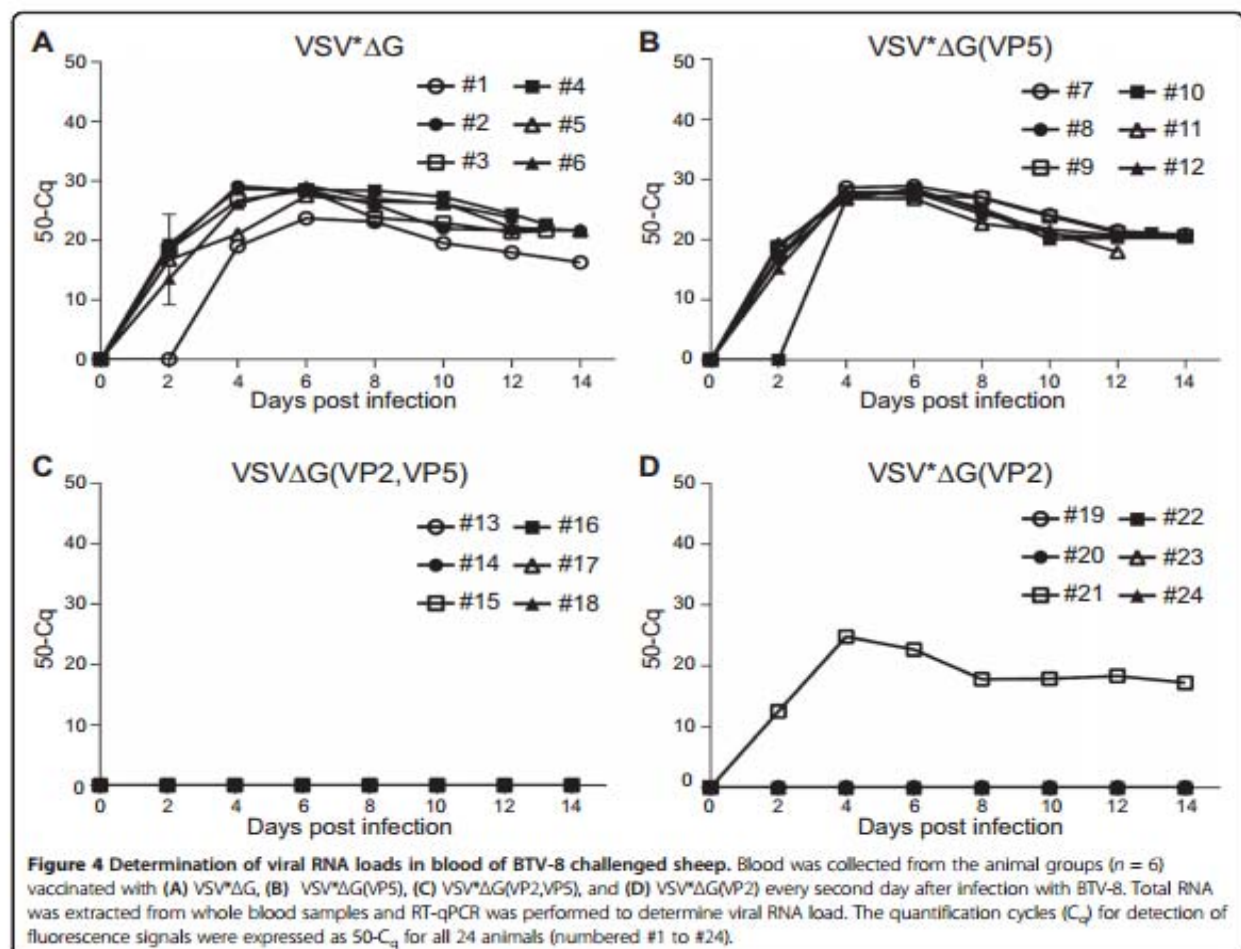
VSV*ΔG(VP5), VSVΔG(VP2,VP5), or VSV*ΔG. Adjuvants were not employed. After 3 weeks, the animals were immunized a second time with the same VRPs. No adverse side effects following vaccination were observed. Sera were analyzed 3 weeks after the second immunization for the presence of BTV-specific antibodies by immunofluorescence. Sera from sheep immunized with either VSV*ΔG(VP2), VSV*ΔG(VP5) or VSVΔG(VP2,VP5) reacted specifically with BTV-8 infected cells, indicating that all recombinant antigens were immunogenic and induced the production of BTV-8 specific antibodies (Figure 2A). Sera from VSV*ΔG-immunized sheep did not react.

The sheep immune sera were tested for neutralizing activity against BTV-8. Pre-immune sera did not show any neutralizing activity confirming that the animals had no pre-existing immunity to BTV-8 (Figure 2B). Sera collected 3 weeks after the first immunization showed limited neutralizing activity but only at the highest serum concentration used (1:4). However, 3 weeks after the second vaccination with VSV*ΔG(VP2) or VSVΔG

(VP2,VP5), all but one of the animals in the VSV*ΔG(VP2) group had developed high neutralizing antibody titres against BTV-8. The same sera did not neutralize BTV-1 or the VSV vector (data not shown). Sheep immunized with either VSV*ΔG or VSV*ΔG(VP5) did not develop BTV-8 neutralizing antibodies. Taken together, these data demonstrate that although recombinant VRPs induced antibodies against both VP2 and VP5, only anti-VP2 antibodies showed BTV-8 neutralizing activity *in vitro*.

VRPs expressing VP2 antigen protect sheep from challenge with pathogenic BTV-8

To evaluate the capacity of recombinant VRP vaccines to induce protective immunity against pathogenic BTV-8, sheep were inoculated intravenously 3 weeks after the second immunization with erythrocyte suspension from a BTV-8 viremic sheep (C_q value of 18). Sheep immunized with VSV*ΔG or VSV*ΔG(VP5) showed characteristic signs of bluetongue disease beginning at day 5 pi (Figure 3A).



Typical symptoms were facial edema, nasal discharge, and lethargy. Three animals from the VSV*ΔG group and two animals from the VSV*ΔG(VP5) group were euthanized for humane reasons at days 12 and 13 pi, respectively, as they developed severe lung edema and respiratory distress. All animals of the two groups developed high fever, which peaked at day 8 after infection (Figure 3B). In contrast, all animals immunized with VSVΔG(VP2,VP5) and 5 out of 6 animals immunized with VSV*ΔG(VP2) did not show any clinical signs of disease (Figure 3A) and had normal rectal temperature (<39.5 °C) throughout the experiment (Figure 3B). These differences were significant between 7 and 11 dpi in the clinical scoring and between 5 and 9 dpi for body temperature. The single animal in the VP2 group, which did not develop a detectable neutralizing antibody titre to BTV (see Figure 2B), showed moderate clinical signs of disease and fever for three days.

The capacity of the vaccine-induced immune response to suppress viremia was evaluated by determining viral RNA loads in blood by quantitative RT-qPCR (Figure 4). Animals immunized with either VSV*ΔG or VSV*ΔG(VP5) had high levels of viral RNA in blood, starting from day 2 post challenge and lasting until the end of the experiment at day 14, whereas BTV RNA was not detected in the blood of the 6 sheep immunized with VSVΔG(VP2, VP5). Likewise, 5 out of 6 sheep immunized with VSV*ΔG(VP2) did not develop any viremia. Thus, recombinant VRPs expressing VP2 alone or in combination with VP5 conferred protective immunity in sheep that prevented both viremia and development of disease.

VP7 antibodies used for differentiation of infected from vaccinated animals

At day 14 after infection, the animals were euthanized and sera tested for the presence of VP7-specific antibodies using a commercially available competitive ELISA (Figure 5). All animals seroconverted following challenge infection irrespective of the vaccine group tested, indicating that even in protected animals (groups VSV*ΔG(VP2) and VSV*ΔG(VP2,VP5)) limited replication of challenge virus must have occurred, which was sufficient to trigger an immune response against VP7. In contrast, all immunized sheep were tested negative for VP7 antibodies prior to infection, indicating that the VRP vaccine fully complied with the DIVA principle.

Discussion

The BTV outer capsid proteins VP2 and VP5 provide potential targets for neutralizing antibodies with all neutralizing epitopes recognized to date residing on VP2. The present study has demonstrated that recombinant VSV replicon particles expressing the VP2 protein of BTV-8 induce the production of serotype-specific neutralizing antibodies that protect sheep from disease and

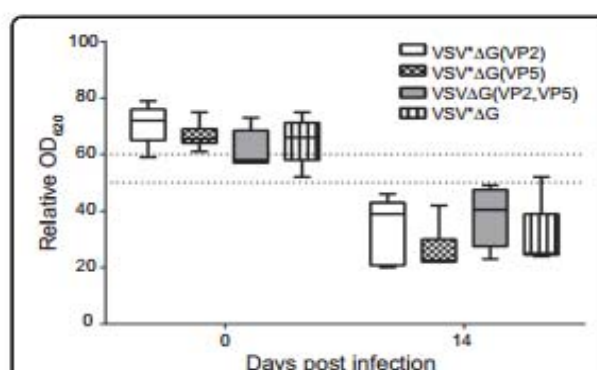


Figure 5 Detection of VP7 antibodies in serum of immunized sheep before and after challenge infection with BTV-8.

Blood was collected from sheep three weeks after the second immunization (prior to challenge infection with BTV-8) and two weeks after challenge by the end of the experiment. Serum antibodies specific for the VP7 antigen of BTV were detected using a commercially available competitive ELISA. Optical density was recorded at 620 nm and expressed as percentage OD₆₂₀ relative to an internal negative control. OD₆₂₀ values < 50% were considered positive, values between 50% and 60% were regarded as doubtful, and values higher than 60% as negative (thresholds indicated by dashed lines).

viremia following challenge infection with BTV-8. Our data confirm that immunization with VP2 alone is sufficient to induce a protective immune response in vaccinated animals [14,33,34]. VP5 has been reported to enhance the protective immune response if present in the vaccine along with VP2 [16,35]. Employing a canarypox virus vector expressing VP2 and VP5 of BTV-17, protection in sheep was achieved, but the relative contributions of VP2 and VP5 to protection have not been addressed [36]. In a recent study, expression of both VP2 and VP5 by a recombinant equine herpesvirus vector was necessary to protect mice against BTV-8 infection, whereas VP2 alone was not fully protective [37]. In our study, VRP co-expressing VP2 and VP5 did not induce higher neutralizing antibody titers than VRP expressing VP2. However, one animal in the VSV*ΔG(VP2) group did not develop neutralizing antibodies and was not protected, whereas all animals in the VSV*ΔG(VP2,VP5) group remained completely healthy following challenge with BTV-8. The question whether this higher protection rate is attributable to the co-expression of VP2 and VP5 should be addressed by additional experiments with a larger number of animals. A critical parameter for the efficacy of vector vaccines could be the level of expression and conformation of the recombinant VP2 antigen. If expression levels are low, the proportion of properly folded VP2 might increase in the presence of VP5 given their extensive conformational interaction [13]. However, if high expression levels of VP2 are achieved with a

given vector system, the amounts of correctly folded VP2 might be sufficient to induce neutralizing antibodies. Antibodies directed to VP5 may not have neutralizing properties on their own, probably because this antigen is not readily accessible for antibodies on the virion surface. Accordingly, the VP5 primary sequence is much more conserved than that of VP2, indicating that VP5 is subject to less immune pressure.

Conventional BTV vaccines have to be properly inactivated and formulated with adjuvants before they can be applied to animals once (sheep) or twice (cattle). Neutralizing antibodies were shown to persist up to four years after immunization [38,39]. The present VRP vaccine did not depend on adjuvants to induce a protective immune response in sheep, probably because this RNA virus vector is cytotoxic and provides sufficient innate signaling to stimulate the immune system. Any adverse side effects which could be caused by adjuvants [40] are thereby eliminated. However, significant levels of neutralizing serum antibodies were detected only after the animals had been vaccinated with the VRP vaccine twice. Additional studies are required to determine the protective effect of a single immunization. In addition, it will be interesting to see for how long VRP-induced neutralizing antibodies will persist in sheep.

Despite their efficacy, live-attenuated BTV vaccines have inherent disadvantages including transmission to insect vectors, lack of attenuation, reassortment of gene segments with field strains of the virus, and potential to cross the placenta to cause reproductive losses and teratogenic effects [41]. In contrast, the propagation-defective VRP vaccine does not cause disease nor will it be able to revert to virulence. As the G protein, the major antigen of VSV, is not expressed by VRPs, neutralizing antibodies against the viral vector are not induced. Accordingly, VRP vaccines are effective when used repeatedly [25,26,42]. Since antibodies directed to VSV G protein are lacking, vaccinated animals will appear seronegative in VSV diagnostic tests [43].

Vector vaccines are superior to conventional vaccines, since they can serve as marker vaccines [22]. The VRP vaccine did not contain the VP7 antigen and therefore allowed the discrimination of infected from vaccinated animals using a commercially available VP7 ELISA. In contrast, conventional (inactivated) BTV vaccines, live-attenuated and propagation-defective BTV vaccines [44,45] do not comply with the DIVA principle. This has complicated the serological surveillance of BTV in those European countries that implemented a vaccination campaign to control the BTV-8 outbreak in 2006. Moreover, the inability to discriminate between infected and vaccinated animals had a significant impact on economy due to restrictions on animal trade [46]. The recombinant replicon particles eliminate this problem as they can be used as marker vaccines.

Currently, there is no universal vaccine available that would protect against all 26 known serotypes of BTV. The VRP technology is not restricted to BTV-8 antigens but may be used to express VP2 antigen of other BTV serotypes as well. Thus, in case a new serotype emerges in a non-endemic region, a recombinant VRP vaccine matching this serotype could be rapidly produced and used for emergency vaccination. This should be particularly valuable for BTV strains for which inactivated vaccines are not readily available because the corresponding viruses do not propagate well in cell culture. Furthermore, this generic vaccine platform may be also employed to protect horses against infection with African horse sickness virus, an orbivirus of which 9 different serotypes are known.

In summary, this study has demonstrated that propagation-incompetent VSV replicon particles can efficiently protect a natural host against bluetongue disease and viremia. This safe and adjuvant-free vaccine technology complies with the DIVA principle, can be easily adapted to other serotypes and viruses, and is rapidly available in case of an emerging BTV outbreak.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SK performed practical and writing work throughout the study in partial fulfillment of the requirements for the D.V.M.-Ph.D. degree from the University of Berne (Graduate School for Cellular and Biomedical Sciences). NR participated in serum neutralization assays, RT-qPCR and ELISA. MH contributed to study design, data analysis and manuscript editing. GZ conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank Daniel Brechbühl and Veronica Ayala for assistance with the animal trials. We are grateful to Dr Malte Dauber (FLI Riems, Germany) for providing mAb 13C10 and Dr Bernd Hoffmann (FLI Riems, Germany) for providing BTV-8 positive blood. We gratefully acknowledge Prof. James N. MacLachlan (UC Davis, USA) for his support throughout the project and for critical reading of this manuscript. We are thankful to Dr Giuseppe Bertoni, Prof. Eliane Marti (Vetsuisse Bern, Switzerland) and Dr Christian Griot (IVI Mittelhäusern, Switzerland) for help and support. This project was funded by Bundesamt für Lebensmittelsicherheit und Veterinärwesen (project number 1.1.1.15).

Received: 19 March 2014 Accepted: 22 May 2014

Published: 13 June 2014

References

1. Borden EC, Shope RE, Murphy FA: Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus—a new taxonomic group. *Physicochemical and serological studies. J Gen Virol* 1971, **13**:261–271.
2. Murphy FA, Borden EC, Shope RE, Harrison A: Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus—a new taxonomic group. *Electron microscopic studies. J Gen Virol* 1971, **13**:273–288.
3. Schwartz-Cornil I, Mertens PP, Contreras V, Hemati B, Pascale F, Breard E, Mellor PS, MacLachlan NJ, Zientara S: Bluetongue virus: virology, pathogenesis and immunity. *Ver Res* 2008, **39**:46.
4. Worwa G, Thur B, Griot C, Hofmann M, MacLachlan JN, Chaignat V: Bluetongue disease in Swiss sheep breeds: clinical signs after experimental infection with bluetongue virus serotype B. *Schweiz Arch Tierheilkd* 2008, **150**:491–498 (in German).

5. Santman-Berends IM, van Wuijkhuise L, Vellema P, van Rijn PA: Vertical transmission of bluetongue virus serotype 8 virus in Dutch dairy herds in 2007. *Ver Microbiol* 2010, **141**:31–35.
6. Saegeman C, Bolkaerts B, Baricalla C, Raes M, Wiggers L, de Leeuw I, Vandebussche F, Zimmer JY, Haubridge E, Cassart D, De Clercq K, Kirschvink N: The impact of naturally-occurring, trans-placental bluetongue virus serotype-8 infection on reproductive performance in sheep. *Ver J* 2011, **187**:72–80.
7. Housawi FM, Abu Elzein EM, Ramadan RD, Gemeel AA, Al-Afaleq AI, Al-Mousa J: Abortions, stillbirths and deformities in sheep at the Al-Ahsa oasis in eastern Saudi Arabia: isolation of a bluetongue serogroup virus from the affected lambs. *Rev Sci Tech* 2004, **23**:913–920.
8. Wouda W, Peperkamp NH, Roumen MP, Muskens J, van Rijn A, Vellema P: Epizootic congenital hydranencephaly and abortion in cattle due to bluetongue virus serotype 8 in the Netherlands. *Tijdschr Diergeneesk* 2009, **134**:422–427.
9. Belhouche M, Mohd Jaafar F, Firth AE, Grimes JM, Mertens PP, Attoui H: Detection of a fourth orbivirus non-structural protein. *PLoS One* 2011, **6**:e25697.
10. Mertens PP, Brown F, Sangar DV: Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology* 1984, **135**:207–217.
11. Mertens PP, Diprose J, Maan S, Singh KP, Attoui H, Samuel AR: Bluetongue virus replication, molecular and structural biology. *Ver Ital* 2004, **40**:426–437.
12. Hassan SS, Roy P: Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *J Virol* 1999, **73**:9832–9842.
13. Zhang X, Boyce M, Bhattacharya B, Zhang X, Schein S, Roy P, Zhou ZH: Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VPS resembles enveloped virus fusion proteins. *Proc Natl Acad Sci U S A* 2010, **107**:6292–6297.
14. Huisman H, van der Walt NT, Cloete M, Erasmus BJ: Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology* 1987, **157**:172–179.
15. DeMaula CD, Bonneau KR, MacLachlan NJ: Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies. *Virus Res* 2000, **67**:59–66.
16. Roy P, Marshall JJ, French TJ: Structure of the bluetongue virus genome and its encoded proteins. *Curr Top Microbiol Immunol* 1990, **162**:43–87.
17. Boyer TC, Ward MP, Wallace RL, Singer RS: Regional seroprevalence of bluetongue virus in cattle in Illinois and western Indiana. *Am J Vet Res* 2007, **68**:1212–1219.
18. MacLachlan NJ, Guthrie AJ: Re-emergence of bluetongue, African horse sickness, and other orbivirus diseases. *Ver Res* 2010, **41**:35.
19. Wilson AJ, Mellor PS: Bluetongue in Europe: past, present and future. *Philos Trans R Soc Lond B Biol Sci* 2009, **364**:2669–2681.
20. MacLachlan NJ, Osburn BI: Impact of bluetongue virus infection on the international movement and trade of ruminants. *J Am Vet Med Assoc* 2006, **228**:1346–1349.
21. Planzer J, Kaufmann C, Worwa G, Gavier-Widen D, Hofmann MA, Chagnat V, Thur B: In vivo and in vitro propagation and transmission of Toggenburg orbivirus. *Res Ver Sci* 2011, **91**:e163–e168.
22. Calvo-Pinilla E, Castillo-Olivares J, Jabbar T, Ortega J, de la Poza F, Marin-Lopez A: Recombinant vaccines against bluetongue virus. *Virus Res* 2014, **182**:78–86.
23. Hastie E, Cataldi M, Mariotti I, Grzelishvili VZ: Understanding and altering cell tropism of vesicular stomatitis virus. *Virus Res* 2013, **176**:16–32.
24. Lichty BD, Power AT, Stojiljkovic DF, Bell JC: Vesicular stomatitis virus: re-inventing the bullet. *Trends Mol Med* 2004, **10**:210–216.
25. Kalishor NH, Veits J, Rautenschlein S, Zimmer G: A recombinant vesicular stomatitis virus replicon vaccine protects chickens from highly pathogenic avian influenza virus (H7N1). *Vaccine* 2009, **27**:1174–1183.
26. Halbherr SJ, Brostoff T, Tippenhauer M, Locher S, Berger Rentsch M, Zimmer G: Vaccination with recombinant RNA replicon particles protects chickens from H5N1 highly pathogenic avian influenza virus. *PLoS One* 2013, **8**:e66059.
27. Hanika A, Larisch B, Steinmann E, Schwegmann-Wessels C, Herler G, Zimmer G: Use of influenza C virus glycoprotein HEF for generation of vesicular stomatitis virus pseudotypes. *J Gen Virol* 2005, **86**:1455–1465.
28. Orru G, Ferrando ML, Meloni M, Licardi M, Savini G, De Santis P: Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. *J Virol Methods* 2006, **137**:34–42.
29. Berger Rentsch M, Zimmer G: A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon. *PLoS One* 2011, **6**:e25858.
30. Worwa G, Hilbe M, Chagnat V, Hofmann MA, Griot C, Ehrensperger F, Doherr MG, Thur B: Virological and pathological findings in Bluetongue virus serotype 8 infected sheep. *Ver Microbiol* 2010, **144**:264–273.
31. Worwa G, Chagnat V, Feldmann J, Thur B: Detection of neutralizing antibodies against Bluetongue virus serotype 8 by an optimized plasma neutralization test. *J Virol Methods* 2013, **188**:168–174.
32. Hoffmann M, Wu YJ, Gerber M, Berger-Rentsch M, Heimrich B, Schwemmler M, Zimmer G: Fusion-active glycoprotein G mediates the cytotoxicity of vesicular stomatitis virus M mutants lacking host shut-off activity. *J Gen Virol* 2010, **91**:2782–2793.
33. Top S, Fournier G, Deplanche M, Rives G, Calvalido J, Cornet L, Bertagnoli S, Meyer G: Myxomavirus as a vector for the immunisation of sheep: protection study against challenge with bluetongue virus. *Vaccine* 2012, **30**:1609–1616.
34. Jabbar TK, Calvo-Pinilla E, Mateos F, Gubbins S, Bin-Tarif A, Bachanek-Bankowska K, Alpar O, Ortega J, Takamatsu HH, Mertens PP, Castillo-Olivares J: Protection of IFNAR (–/–) mice against bluetongue virus serotype 8, by heterologous (DNA/rMVA) and homologous (rMVA/rMVA) vaccination, expressing outer-capsid protein VP2. *PLoS One* 2013, **8**:e60574.
35. Lobato ZI, Coupar BE, Gray CP, Lunt R, Andrew ME: Antibody responses and protective immunity to recombinant vaccinia virus-expressed bluetongue virus antigens. *Ver Immunol Immunopathol* 1997, **59**:293–309.
36. Boone JD, Balasuriya UB, Karaca K, Audonnet JC, Yao J, He L, Nordgren R, Monaco F, Savini G, Gardner IA, MacLachlan NJ: Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VPS outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine* 2007, **25**:672–678.
37. Ma G, Eschbaumer M, Said A, Hoffmann B, Beer M, Osterrieder N: An equine herpesvirus type 1 (EHV-1) expressing VP2 and VPS of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model. *PLoS One* 2012, **7**:e34425.
38. Oura CA, Edwards L, Batten CA: Evaluation of the humoral immune response in adult dairy cattle three years after vaccination with a bluetongue serotype 8 inactivated vaccine. *Vaccine* 2012, **30**:112–115.
39. Batten CA, Edwards L, Oura CA: Evaluation of the humoral immune responses in adult cattle and sheep, 4 and 2.5 years post-vaccination with a bluetongue serotype 8 inactivated vaccine. *Vaccine* 2013, **31**:3783–3785.
40. Gonzalez JM, Figueras L, Ortega ME, Lozano M, de Arcaute MR, Royo R, Cebrian LM, Ferrer LM, Farinas F, de Jalon JA, De las Heras M: Possible adverse reactions in sheep after vaccination with inactivated BTV vaccines. *Ver Rec* 2010, **166**:757–758.
41. Zientara S, MacLachlan NJ, Calistri P, Sanchez-Vizcaino JM, Savini G: Bluetongue vaccination in Europe. *Expert Rev Vaccines* 2010, **9**:989–991.
42. Schwartz JA, Buonocore L, Suguitan A Jr, Hunter M, Marx PA, Subbarao K, Rose JK: Vesicular stomatitis virus-based H5N1 avian influenza vaccines induce potent cross-clade neutralizing antibodies in rhesus macaques. *J Virol* 2011, **85**:4602–4605.
43. Roberts A, Buonocore L, Price R, Forman J, Rose JK: Attenuated vesicular stomatitis viruses as vaccine vectors. *J Virol* 1999, **73**:3723–3732.
44. Matsuo E, Celma CC, Boyce M, Viarouge C, Sailleau C, Dubois E, Breard E, Thiery R, Zientara S, Roy P: Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. *J Virol* 2011, **85**:10213–10221.
45. Celma CC, Boyce M, van Rijn PA, Eschbaumer M, Wemike K, Hoffmann B, Beer M, Haegeman A, De Clercq K, Roy P: Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. *J Virol* 2013, **87**:9856–9864.
46. Savini G, MacLachlan NJ, Sanchez-Vizcaino JM, Zientara S: Vaccines against bluetongue in Europe. *Comp Immunol Microbiol Infect Dis* 2008, **31**:101–120.

doi:10.1186/1729-9716-45-64

Cite this article as: Kochinger et al.: Vesicular stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus serotype 8 induces complete protection of sheep against challenge infection. *Veterinary Research* 2014 **45**:64.

CONCLUSION AND OUTLOOK

The BTV outer capsid proteins VP2 and VP5 represent potential targets for neutralizing antibodies. However, all neutralizing epitopes identified to date reside on VP2, and VP5 is thought to exert a conformational effect on VP2 (26). The present study demonstrates that recombinant VRPs that express the VP2 protein of BTV-8 induce the production of serotype-specific neutralizing antibodies that protect sheep from disease and viremia following challenge infection with BTV-8. Our data confirm previous reports that have demonstrated that immunization with VP2 alone is sufficient to induce a protective immune response [25, 62, 63]. VP5 has been reported to enhance the protective immune response if present in the vaccine along with VP2 [27, 38]. Employing a canarypox virus vector expressing VP2 and VP5 of BTV-17, protection in sheep was achieved, but the relative contributions of VP2 and VP5 to protection have not been addressed [39]. In a recent study, expression of both VP2 and VP5 by a recombinant equine herpesvirus vector was necessary to protect mice against BTV-8 infection, whereas VP2 alone was not fully protective [41]. In our investigations, VRPs co-expressing VP2 and VP5 did not induce higher neutralizing antibody titers than VRP expressing only VP2, although one animal immunized with VP2 was not protected nor did it develop neutralizing antibodies. This discrepancy between VSV-based VRPs and other vaccines might be due to the different vector systems, i.e. different expression levels and differences in the conformation of recombinant VP2, especially if yields of recombinant VP2 are low. Thus, the proportion of properly folded VP2 might increase in the presence of VP5 given their extensive conformational interaction [24]. If high expression levels of VP2 can be achieved with a given vector system, the amounts of correctly folded VP2 might be sufficient to induce neutralizing antibodies. Antibodies directed to VP5 may

not have neutralizing properties on their own, probably because this protein is not accessible for antibodies on the surface of the virion. Furthermore, VP5 is more conserved than VP2, suggesting that VP5 is subject to less immune pressure.

Conventional BTV vaccines have to be properly inactivated and formulated with adjuvants before they can be applied to animals once (sheep) or twice (cattle). Neutralizing antibodies were shown to persist up to four years after immunization [64, 65]. The present VRP vaccine did not depend on adjuvants to induce a protective immune response in sheep, probably because the RNA virus vector is cytotoxic and so stimulates the innate immune system via pathogen recognition receptor. Any adverse side effects that could be caused by adjuvants [66] are thereby eliminated. However, significant levels of neutralizing serum antibodies were detected only after the animals had been vaccinated with the VRP vaccine twice. Additional studies are required to determine the protective effect of a single immunization. In addition, it will be interesting to see for how long VRP-induced neutralizing antibodies will persist in sheep.

Despite their efficacy, MLV (live-attenuated) BTV vaccines have inherent disadvantages, including potential transmission to vector midges, lack of attenuation, reassortment of gene segments with field strains of the virus, and potential to cross the placenta to cause reproductive losses and teratogenesis [67]. In contrast, the propagation-defective VRP vaccine does not cause disease nor will it be able to revert to virulence. As the VSV G protein which is the major immunogenic VSV protein is not expressed neither by the VRPs nor in non-helper cells, antibodies directed against the viral vector are not induced. Accordingly, VRP vaccines are effective when used repeatedly [51, 52, 68]. Since antibodies directed to VSV G

protein are lacking, vaccinated animals will appear seronegative in VSV neutralization tests [69].

Vector vaccines are superior to conventional vaccines, since they can serve as marker vaccines [42]. The VRP vaccine did not contain the VP7 antigen and therefore allowed the discrimination of infected from vaccinated animals using a commercially available BTV VP7 ELISA. In contrast, conventional (inactivated) BTV vaccines, live-attenuated and propagation-defective BTV vaccines [70, 71] do not comply with the DIVA principle. This has complicated the serological surveillance of BTV in those European countries that implemented a vaccination campaign to control the BTV-8 outbreak in 2006. Moreover, the inability to discriminate between infected and vaccinated animals had a significant impact on economy due to restrictions on animal trade [31]. The recombinant replicon particles eliminate this problem as they can be used as marker vaccines.

There is no universal vaccine available that would protect against all 26 currently known serotypes of BTV. The VRP technology is not restricted to BTV-8 antigens but can be also used to express VP2 antigen of other BTV serotypes. Thus, in the scenario where a new BTV serotype emerges in a non-endemic region, a recombinant VRP vaccine matching this serotype could be rapidly produced and used for emergency vaccination. This should be particularly valuable for BTV serotypes and strains for which inactivated vaccines are not readily available because the corresponding viruses do not propagate well in cell culture. Furthermore, this generic vaccine platform may be also be relevant to protect horses against infection with African horse sickness virus. In summary, this study demonstrates that propagation-incompetent VSV replicon particles can efficiently protect a natural host against BT disease and viremia. This safe and adjuvant-free

vaccine technology complies with the DIVA principle, can be easily adapted to other serotypes or other viruses, and is rapidly available in case of an emerging BTV outbreak.

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my advisor **Gert Zimmer** for the help throughout my PhD study and research. My sincere thanks also goes to my co-advisor **Eliane Marti** for encouragement, insightful comments and help.

I want to thank external Co-advisor, **Jim MacLachlan** for sharing his knowledge, wisdom and experience with me.

Besides my advisors, I would like to thank my excellent mentor **Giuseppe Bertoni** for continuous support, help and for being a person who I really learned to admire, both for his knowledge and his personal resilience.

I thank the Institute of Virology and Immunology, **Christian Griot**, **Kathrin Summermatter**, **Martin Hofmann**, **Nahalie Renevey**, **Barbara Thür**, **Andrea Vögtlin**, **Lukas Bruckner**, **Volker Thiel**, **Arthur Summerfield**, **Meret Ricklin**, and all my fellow lab mates for their support and motivation throughout the past three years.

I also thank my dear Austrian (**Barbara Matzinger**, **Georg Schätz**, **Sonja Prucha**, **Viviane Benetka** and **Verena Küronya**) and Swiss (**Patricia Neupert**, **Lena Ferner**, **Claudia Wehinger**, **Roger Büchi**, **Tobias Meyer**, **Tobias Struchen** and **Daniela Juen**) friends for all the fun, time and love you gave me. Friends like you make the world go round!

Furthermore, I would like to thank my beloved family-first and foremost my gorgeous **mother Christa**, my sporty **father Günther**, my adventurous **brother Christian**, my caring **sister Tanja** and my great **aunt Luise** for raising me, teaching me and loving me the way they did and do throughout my life. I could not think of a better family!

I also thank my boyfriend's parents **Regula and Florian Meyer** for the nice family dinners at their place and **Samuel** for being both, my best friend and love. I truly admire, appreciate and love you just the way you are!

Last but not least, I thank my Irish Setter dog **Diana** for being an example of patience and sweetness.

CURRICULUM VITAE

CONTACT DETAILS



STEFANIE KOCHINGER

Zollikon, Switzerland

Fohrbachstrasse 16 • 8702 Zollikon • Switzerland
+41 79 1247980 • stefanie.kochinger@gmx.at
June 27, 1986

PROFESSIONAL EXPERIENCE

INSTITUTE OF VIROLOGY AND IMMUNOLOGY

Mittelhäusern, Switzerland

Development of a novel marker vaccine platform against Bluetongue

August 2011 – Juli 2014

- Execution of BLV-granted project
- Worked in a BSL-4 facility
- Conducted reverse genetics, serology, animal trials, qRT-PCR, cell culture, pathology
- Participation and presentations in national and international conferences

INSTITUTE OF ANIMAL WELFARE

Vienna, Austria

Research Intern

February 2011 – May 2011

- Evaluated breeding facilities for pigs and chickens

CLINICAL VIROLOGY

Vienna, Austria

Diagnostics and research assistant

October 2010 – Juli 2011

- Virological and serological testing of samples
- Helped implementing QM-system for certification (GLP, ISO)
- Project: Canine Parvovirus type 2; evaluation of a novel qPCR for diagnostic use
- Teaching and supervision of students

AGES INSTITUTE OF FOOD SAFETY

Vienna, Austria

Intern

- Laboratory diagnostics milk and meat

September 2011

AGES PHARMMED

Vienna, Austria

Intern Registration of human therapeutics

July 2009 – August 2009

- Wrote and corrected expert opinions on human therapeutics

EDUCATION

FEDERAL FOOD SAFETY AND VETERINARY OFFICE **Berne, Switzerland**
Federal veterinary officer studies 2014

GRADUATE SCHOOL FOR CELLULAR AND MICROBIOLOGICAL SCIENCE **Berne, Switzerland**
DVM-Ph.D. 2011-2014

SVVLD - SCHWEIZER VEREINIGUNG DER VETERINÄR-LABORDIAGNOSTIKER **Berne, Switzerland**
Fachtierarzt FVH für Labor- und Grundlagenmedizin, Virologie 2011-2014

UNIVERSITY OF VETERINARY MEDICINE **Vienna, Austria**
Veterinary Medicine 2004-2011

- Completed study in minimum required time with excellent success
- Specialized in Public Health and was intern in different divisions

GYMNASIUM DER DOMINIKANERINNEN **Vienna, Austria**
Matura 1996 – 2004

PERSONAL

LANGUAGES

English: fluent • **German:** fluent (native) • **French:** basic

TOOLS

Microsoft **Office** • Prism**Graph** • Adobe **Illustrator CS6** • Adobe **Photoshop Elements 10**

INTERESTS

Jogging and walking with my dog • **Meeting** with colleagues • **Horseback riding**
Exploring new places • **Skiing** in winter • **Reading** books and papers

LIST OF PUBLICATIONS

Kochinger S., Renevey N., Hofmann M. A., Zimmer, G. 2014. Vesicular stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus serotype 8 induces complete protection of sheep against challenge infection. *Vet Res* 2014, 45:64.

DECLARATION OF ORIGINALITY

Last name, first name: KOCHINGER, Stefanie

Matriculation number: 11-127-297

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Mittelhäusern, 01.06.2014


Signature

REFERENCES

1. Vet Immunol Immunopathol DNA RaHtDaT, Prof. Anica Dricu (Ed.) Erasmus, B. J.: **Bluetongue in sheep and goats.** *Aust Vet J* 1975, **51**:165-170.
2. van der Sluijs MT, Schroer-Joosten DP, Fid-Fourkour A, Vrijenhoek MP, Debyser I, Moulin V, Moormann RJ, de Smit AJ: **Transplacental transmission of bluetongue virus serotype 1 and serotype 8 in sheep: virological and pathological findings.** *PloS one* 2013, **8**:e81429.
3. Santman-Berends IM, van Wuijckhuise L, Vellema P, van Rijn PA: **Vertical transmission of bluetongue virus serotype 8 virus in Dutch dairy herds in 2007.** *Vet Microbiol* 2010, **141**:31-35.
4. Schwartz-Cornil I, Mertens PP, Contreras V, Hemati B, Pascale F, Breard E, Mellor PS, MacLachlan NJ, Zientara S: **Bluetongue virus: virology, pathogenesis and immunity.** *Vet Res* 2008, **39**:46.
5. Mellor PS, Boorman J: **The transmission and geographical spread of African horse sickness and bluetongue viruses.** *Ann Trop Med Parasitol* 1995, **89**:1-15.
6. Tabachnick WJ: **Culicoides variipennis and bluetongue-virus epidemiology in the United States.** *Annu Rev Entomol* 1996, **41**:23-43.
7. Mellor PS, Wittmann EJ: **Bluetongue virus in the Mediterranean Basin 1998-2001.** *Vet J* 2002, **164**:20-37.
8. MacLachlan NJ, Guthrie AJ: **Re-emergence of bluetongue, African horse sickness, and other orbivirus diseases.** *Vet Res* 2010, **41**:35.
9. Wilson AJ, Mellor PS: **Bluetongue in Europe: past, present and future.** *Philos Trans R Soc Lond B Biol Sci* 2009, **364**:2669-2681.
10. MacLachlan NJ, Osburn BI: **Impact of bluetongue virus infection on the international movement and trade of ruminants.** *J Am Vet Med Assoc* 2006, **228**:1346-1349.
11. Rasmussen LD, Rasmussen TB, Belsham GJ, Strandbygaard B, Botner A: **Bluetongue in Denmark during 2008.** *Vet Rec* 2010, **166**:714-718.
12. Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ: **Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes.** *Rev Sci Tech* 2008, **27**:427-442.

13. Saegerman C, Berkvens D, Mellor PS: **Bluetongue epidemiology in the European Union.** *Emerg Infect Dis* 2008, **14**:539-544.
14. Worwa G, Thur B, Griot C, Hofmann M, MacLachlan JN, Chaignat V: **[Bluetongue disease in Swiss sheep breeds: clinical signs after experimental infection with bluetongue virus serotype 8].** *Schweiz Arch Tierheilkd* 2008, **150**:491-498.
15. Barratt-Boyes SM, MacLachlan NJ: **Dynamics of viral spread in bluetongue virus infected calves.** *Vet Microbiol* 1994, **40**:361-371.
16. Saegerman C, Bolkaerts B, Baricalla C, Raes M, Wiggers L, de Leeuw I, Vandenbussche F, Zimmer JY, Haubruge E, Cassart D, et al: **The impact of naturally-occurring, trans-placental bluetongue virus serotype-8 infection on reproductive performance in sheep.** *Vet J* 2011, **187**:72-80.
17. Housawi FM, Abu Elzein EM, Ramadan RO, Gameel AA, Al-Afaleq AI, Al-Mousa J: **Abortions, stillbirths and deformities in sheep at the Al-Ahsa oasis in eastern Saudi Arabia: isolation of a bluetongue serogroup virus from the affected lambs.** *Rev Sci Tech* 2004, **23**:913-920.
18. Wouda W, Peperkamp NH, Roumen MP, Muskens J, van Rijn A, Vellema P: **Epizootic congenital hydranencephaly and abortion in cattle due to bluetongue virus serotype 8 in the Netherlands.** *Tijdschr Diergeneeskd* 2009, **134**:422-427.
19. Verwoerd DW, Louw H, Oellermann RA: **Characterization of bluetongue virus ribonucleic acid.** *J Virol* 1970, **5**:1-7.
20. Belhouchet M, Mohd Jaafar F, Firth AE, Grimes JM, Mertens PP, Attoui H: **Detection of a fourth orbivirus non-structural protein.** *PloS one* 2011, **6**:e25697.
21. Mertens PP, Brown F, Sangar DV: **Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode.** *Virology* 1984, **135**:207-217.
22. Mertens PP, Diprose J, Maan S, Singh KP, Attoui H, Samuel AR: **Bluetongue virus replication, molecular and structural biology.** *Vet Ital* 2004, **40**:426-437.
23. Hassan SS, Roy P: **Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry.** *J Virol* 1999, **73**:9832-9842.
24. Zhang X, Boyce M, Bhattacharya B, Zhang X, Schein S, Roy P, Zhou ZH: **Bluetongue virus coat protein VP2 contains sialic acid-binding domains,**

and VP5 resembles enveloped virus fusion proteins. *Proc Natl Acad Sci U S A* 2010, **107**:6292-6297.

25. Huismans H, van der Walt NT, Cloete M, Erasmus BJ: **Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep.** *Virology* 1987, **157**:172-179.
26. DeMaula CD, Bonneau KR, MacLachlan NJ: **Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies.** *Virus Res* 2000, **67**:59-66.
27. Roy P, Marshall JJ, French TJ: **Structure of the bluetongue virus genome and its encoded proteins.** *Curr Top Microbiol Immunol* 1990, **162**:43-87.
28. Hassan SH, Wirblich C, Forzan M, Roy P: **Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization.** *J Virol* 2001, **75**:8356-8367.
29. Boyer TC, Ward MP, Wallace RL, Singer RS: **Regional seroprevalence of bluetongue virus in cattle in Illinois and western Indiana.** *Am J Vet Res* 2007, **68**:1212-1219.
30. Patel A, Roy P: **The molecular biology of Bluetongue virus replication.** *Virus Res* 2013.
31. Savini G, MacLachlan NJ, Sanchez-Vizcaino JM, Zientara S: **Vaccines against bluetongue in Europe.** *Comp Immunol Microbiol Infect Dis* 2008, **31**:101-120.
32. Veronesi E, Darpel KE, Hamblin C, Carpenter S, Takamatsu HH, Anthony SJ, Elliott H, Mertens PP, Mellor PS: **Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4.** *Vaccine* 2010, **28**:1397-1403.
33. Batten CA, Maan S, Shaw AE, Maan NS, Mertens PP: **A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment.** *Virus Res* 2008, **137**:56-63.
34. Planzer J, Kaufmann C, Worwa G, Gavier-Widen D, Hofmann MA, Chaignat V, Thur B: **In vivo and in vitro propagation and transmission of Toggenburg orbivirus.** *Res Vet Sci* 2011, **91**:e163-168.
35. Roy P, French T, Erasmus BJ: **Protective efficacy of virus-like particles for bluetongue disease.** *Vaccine* 1992, **10**:28-32.
36. Roy P, Bishop DH, LeBlois H, Erasmus BJ: **Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like**

particles: evidence for homologous and partial heterologous protection. *Vaccine* 1994, **12**:805-811.

37. Hervas-Stubbs S, Rueda P, Lopez L, Leclerc C: **Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN.** *J Immunol* 2007, **178**:2361-2369.
38. Lobato ZI, Coupar BE, Gray CP, Lunt R, Andrew ME: **Antibody responses and protective immunity to recombinant vaccinia virus-expressed bluetongue virus antigens.** *Vet Immunol Immunopathol* 1997, **59**:293-309.
39. Boone JD, Balasuriya UB, Karaca K, Audonnet JC, Yao J, He L, Nordgren R, Monaco F, Savini G, Gardner IA, Maclachlan NJ: **Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep.** *Vaccine* 2007, **25**:672-678.
40. Wade-Evans AM, Romero CH, Mellor P, Takamatsu H, Anderson J, Thevasagayam J, Fleming MJ, Mertens PP, Black DN: **Expression of the major core structural protein (VP7) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge.** *Virology* 1996, **220**:227-231.
41. Ma G, Eschbaumer M, Said A, Hoffmann B, Beer M, Osterrieder N: **An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model.** *PloS one* 2012, **7**:e34425.
42. Calvo-Pinilla E, Castillo-Olivares J, Jabbar T, Ortego J, de la Poza F, Marin-Lopez A: **Recombinant vaccines against bluetongue virus.** *Virus Res* 2013.
43. Hastie E, Cataldi M, Marriott I, Grdzlishvili VZ: **Understanding and altering cell tropism of vesicular stomatitis virus.** *Virus Res* 2013, **176**:16-32.
44. Zhang JLaY: **Messenger RNA Cap Methylation in Vesicular Stomatitis Virus, a Prototype of Non-Segmented Negative-Sense RNA Virus, Methylation.** *DNA, RNA and Histones to Diseases and Treatment, Prof Anica Dricu (Ed)* 2012.
45. Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y: **A system for functional analysis of Ebola virus glycoprotein.** *Proc Natl Acad Sci U S A* 1997, **94**:14764-14769.
46. Kapadia SU, Simon ID, Rose JK: **SARS vaccine based on a replication-defective recombinant vesicular stomatitis virus is more potent than one based on a replication-competent vector.** *Virology* 2008, **376**:165-172.

47. Publicover J, Ramsburg E, Rose JK: **A single-cycle vaccine vector based on vesicular stomatitis virus can induce immune responses comparable to those generated by a replication-competent vector.** *J Virol* 2005, **79**:13231-13238.
48. Lichty BD, Power AT, Stojdl DF, Bell JC: **Vesicular stomatitis virus: re-inventing the bullet.** *Trends Mol Med* 2004, **10**:210-216.
49. Majid AM, Barber GN: **Recombinant Vesicular Stomatitis Virus (VSV) and Other Strategies in HCV Vaccine Designs and Immunotherapy.** In *Hepatitis C Viruses: Genomes and Molecular Biology*. Edited by Tan SL. Norfolk (UK); 2006
50. Kahn JS, Roberts A, Weibel C, Buonocore L, Rose JK: **Replication-competent or attenuated, nonpropagating vesicular stomatitis viruses expressing respiratory syncytial virus (RSV) antigens protect mice against RSV challenge.** *J Virol* 2001, **75**:11079-11087.
51. Kalhoro NH, Veits J, Rautenschlein S, Zimmer G: **A recombinant vesicular stomatitis virus replicon vaccine protects chickens from highly pathogenic avian influenza virus (H7N1).** *Vaccine* 2009, **27**:1174-1183.
52. Halbherr SJ, Brostoff T, Tippenhauer M, Locher S, Berger Rentsch M, Zimmer G: **Vaccination with recombinant RNA replicon particles protects chickens from H5N1 highly pathogenic avian influenza virus.** *PloS one* 2013, **8**:e66059.
53. Berger Rentsch M, Zimmer G: **A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon.** *PloS one* 2011, **6**:e25858.
54. Zimmer G: **RNA Replicons - A New Approach for Influenza Virus Immunoprophylaxis.** *Viruses* 2010, **2**:413-434.
55. Borden EC, Shope RE, Murphy FA: **Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus--a new taxonomic group. Physiocochemical and serological studies.** *J Gen Virol* 1971, **13**:261-271.
56. Murphy FA, Borden EC, Shope RE, Harrison A: **Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus--a new taxonomic group. Electron microscopic studies.** *J Gen Virol* 1971, **13**:273-288.
57. Hanika A, Larisch B, Steinmann E, Schwegmann-Wessels C, Herrler G, Zimmer G: **Use of influenza C virus glycoprotein HEF for generation of vesicular stomatitis virus pseudotypes.** *J Gen Virol* 2005, **86**:1455-1465.

58. Orru G, Ferrando ML, Meloni M, Liciardi M, Savini G, De Santis P: **Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay.** *J Virol Methods* 2006, **137**:34-42.
59. Worwa G, Hilbe M, Chaignat V, Hofmann MA, Griot C, Ehrensperger F, Doherr MG, Thur B: **Virological and pathological findings in Bluetongue virus serotype 8 infected sheep.** *Vet Microbiol* 2010, **144**:264-273.
60. Worwa G, Chaignat V, Feldmann J, Thur B: **Detection of neutralizing antibodies against Bluetongue virus serotype 8 by an optimized plasma neutralization test.** *J Virol Methods* 2013, **188**:168-174.
61. Hoffmann M, Wu YJ, Gerber M, Berger-Rentsch M, Heimrich B, Schwemmler M, Zimmer G: **Fusion-active glycoprotein G mediates the cytotoxicity of vesicular stomatitis virus M mutants lacking host shut-off activity.** *J Gen Virol* 2010, **91**:2782-2793.
62. Top S, Foucras G, Deplanche M, Rives G, Calvalido J, Comtet L, Bertagnoli S, Meyer G: **Myxomavirus as a vector for the immunisation of sheep: protection study against challenge with bluetongue virus.** *Vaccine* 2012, **30**:1609-1616.
63. Jabbar TK, Calvo-Pinilla E, Mateos F, Gubbins S, Bin-Tarif A, Bachanek-Bankowska K, Alpar O, Ortego J, Takamatsu HH, Mertens PP, Castillo-Olivares J: **Protection of IFNAR (-/-) mice against bluetongue virus serotype 8, by heterologous (DNA/rMVA) and homologous (rMVA/rMVA) vaccination, expressing outer-capsid protein VP2.** *PloS one* 2013, **8**:e60574.
64. Oura CA, Edwards L, Batten CA: **Evaluation of the humoral immune response in adult dairy cattle three years after vaccination with a bluetongue serotype 8 inactivated vaccine.** *Vaccine* 2012, **30**:112-115.
65. Batten CA, Edwards L, Oura CA: **Evaluation of the humoral immune responses in adult cattle and sheep, 4 and 2.5 years post-vaccination with a bluetongue serotype 8 inactivated vaccine.** *Vaccine* 2013, **31**:3783-3785.
66. Gonzalez JM, Figueras L, Ortega ME, Lozano M, de Arcaute MR, Royo R, Cebrian LM, Ferrer LM, Farinas F, de Jalon JA, De las Heras M: **Possible adverse reactions in sheep after vaccination with inactivated BTV vaccines.** *Vet Rec* 2010, **166**:757-758.
67. Zientara S, MacLachlan NJ, Calistri P, Sanchez-Vizcaino JM, Savini G: **Bluetongue vaccination in Europe.** *Expert Rev Vaccines* 2010, **9**:989-991.
68. Schwartz JA, Buonocore L, Suguitan A, Jr., Hunter M, Marx PA, Subbarao K, Rose JK: **Vesicular stomatitis virus-based H5N1 avian influenza vaccines**

induce potent cross-clade neutralizing antibodies in rhesus macaques. *J Virol* 2011, **85:4602-4605.**

69. Roberts A, Buonocore L, Price R, Forman J, Rose JK: **Attenuated vesicular stomatitis viruses as vaccine vectors. *J Virol* 1999, **73**:3723-3732.**
70. Matsuo E, Celma CC, Boyce M, Viarouge C, Sailleau C, Dubois E, Breard E, Thiery R, Zientara S, Roy P: **Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. *J Virol* 2011, **85**:10213-10221.**
71. Celma CC, Boyce M, van Rijn PA, Eschbaumer M, Wernike K, Hoffmann B, Beer M, Haegeman A, De Clercq K, Roy P: **Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. *J Virol* 2013, **87**:9856-9864.**